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The Effects of Trypan Blue on Chick Embryos Cultured *in vitro*

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INTRODUCTION

VARIOUS malformations in rats and mice have been observed to be caused by administration of the vital dye, Trypan blue (Gillman, Gilbert, Gillman, & Spence, 1948; Fox & Goss, 1956, 1957; Hamburgh, 1952, 1954; Waddington & Carter, 1952, 1953). Recently Waddington & Perry (1956) reported a teratogenic effect of Trypan blue on Amphibian embryos. The present paper deals with the effects of this dye on cultured chick embryos.

MATERIAL AND METHODS

Hen's eggs were incubated at 38° C. to the desired stage of development. Cultures were set up according to the ring technique described by New (1955). Precautions to maintain sterile conditions were observed throughout. An aqueous 1 per cent. solution of Gurr's vital Trypan blue was diluted with Pannett Compton saline to 0·05 per cent., which had been found to be a suitable concentration. A few drops of the dye solution were placed on the upper surface of the treated blastoderms. The embryos were kept for about half an hour at room temperature before incubating to allow proper diffusion of the dye before development was resumed. The dye was left in contact with the treated embryos throughout the whole culture period.

A total of 28 embryos at the primitive-streak or early head-process stage, and 13 embryos at the medullary-plate stage, were treated and studied. Twenty-six control embryos at the primitive-streak or early head-process stage received a few drops of saline instead of the dye and were maintained under identical conditions. Both the controls and the experimental cultures were incubated for 14–16 hours, after which they were fixed in acetic alcohol and serially sectioned at 10 μ. In some experiments the entire embryos were stained in borax carmine, differentiated in acid alcohol, and sketched before sectioning.

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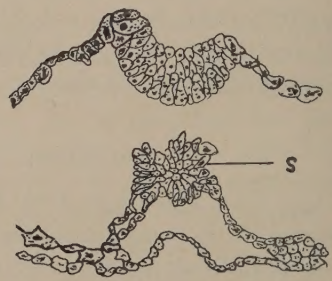
EXPERIMENTAL RESULTS

Of the 28 embryos treated at the primitive-streak or early head-process stage, 18 showed abnormalities. Fifteen of the abnormal embryos showed malformation of the somites. In some regions somitic mesoderm was not formed at all (Text-fig. 2). In others it was formed but the somites were irregular and asymmetrical (Text-fig. 6). Extensive failure of notochord development occurred in 4 specimens.

TEXT-FIG. 1. Embryo treated with dye at the early head-process stage and incubated for a further 16 hours. ×66.

TEXT-FIG. 2. Section through embryo shown in Text-fig. 1. Notochord and somites are absent. ×90.



TEXT-FIG. 3. Section through embryo shown in Text-fig. 1. Somitic mesoderm (*s*) is fused in the middle in the absence of notochord. ×112.

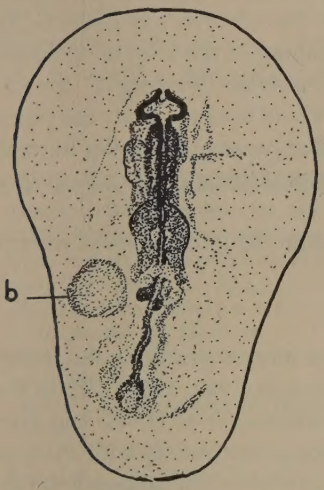
All the drawings were made from photographs.

The embryo illustrated in Text-figs. 1, 2, and 3 was in an early head-process stage when treated. On sectioning it was found that the notochord was absent except in a few anterior sections. The mesoderm showed a tendency towards somite formation and in part there was a median somite strand (Text-fig. 3). The presence of such median somitic mesoderm in the absence of notochord has also been described by Waddington (1932) and Waddington & Perry (1956).

In two cases the brain and the optic vesicles were found to be very small. Shortening of the axis of the embryos was also observed in 9 treated embryos.

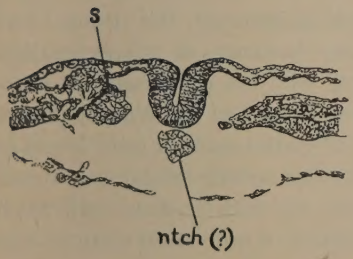
In nine cases the heart did not show its characteristc flexure and was represented by a somewhat dilated straight tube for a considerable time, although the

heart of the control at the same stage showed the characteristic flexure to the right.



TEXT-FIG. 4. Embryo treated at primitive-streak stage showing blister (*b*) over the blastoderm. ×66.

TEXT-FIG. 5. Section of embryo shown in Text-fig. 4, through blister. ×110.



TEXT-FIG. 6. Section showing irregular somite (*s*) formation. The notochord (*ntch.*) appears to be big for the neural plate. It may be a somite on the right side moving towards the centre in the absence of the notochord. ×80.

TEXT-FIG. 7. Embryo treated at the medullary-plate stage showing shortening of the axis and inhibition of somite formation. ×47.

Large transparent blisters were seen in 4 embryos, especially posteriorly between the ectoderm and mesoderm (Text-fig. 4). Sections of the embyro passing

through the blister showed suppression of the neural plate beside the blister, failure of notochord formation, and abnormalities in the somites (Text-fig. 5).

Of 13 embryos treated at the medullary-plate stage 7 showed more or less similar abnormalities. The anterior portion of the embryo appeared to have developed normally in these embryos; but shortening of the axis, malformation of somites, and transparent blisters were quite common (Text-fig. 7). Six of 13 cultures treated at this stage were incubated for 42–44 hours to study the effect of dye on the development of the eye, but the eyes appeared to develop normally. However, in two cases the eye and the nearby neural tissue of the brain showed an unusual amount of cell degeneration.

None of the malformations described were observed in the control embryos.

DISCUSSION

Some of the abnormalities such as transparent blisters overlying the embryo, and abnormal hearts reported for mice by Hamburgh (1952, 1954) and by Waddington & Carter (1952, 1953), and for rats by Gillman *et al.* (1948) and Fox & Goss (1956, 1957), have also been observed in the present study with chick embryos. In both mice and rats ocular defects such as eyelessness (anophthalmia) or absence of lens, &c., seemed to be quite common; but in the chick embryos which were incubated for 42–44 hours for the study of the development of eye no defects were seen, though in two cases the eye showed unusual cell degeneration.

Cases of pseudencephaly have been described in mice at birth by Waddington, but in the present work the embryos were not kept alive long enough for this effect to be observed. In mice and rats the nervous and circulatory systems seemed to be severely affected by the Trypan blue treatment, and minor effects on notochord and somites were also observed; in contrast, the somites and notochord of Amphibia seem to be much affected. Instances of mesodermalization consisting of suppression of the notochord with the conversion of notochord material into somitic material have been described by Waddington & Perry (1956). In the present study the majority of malformations were found in somites. Where notochord failed to appear a median somite strand was found, as observed in Amphibia by Waddington & Perry; but with the data available it cannot be said with certainty that mesodermalization of notochord material had occurred.

SUMMARY

1. Chick embryos at the primitive-streak or early head-process and at the medullary-plate stages were treated with Trypan blue during culture *in vitro*.

2. The dye produced many abnormalities such as malformation of somites, shortening of the axis, the formation of large blisters on the embryo, and delayed heart flexure. In some specimens notochord failed to form and a median somite strand was found in these cases.

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A propos du raccourcissement de la ligne primitive du blastoderme du Poulet

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AVEC UNE PLANCHE

INTRODUCTION

SPRATT (1947) a minutieusement étudié la régression et le raccourcissement de la ligne primitive. Il met très nettement en évidence les relations qui existent entre la régression du nœud de Hensen et la croissance globale du germe; il leur consacre d'ailleurs une série de travaux ultérieurs (1955, 1957*a*, 1957*b*, 1958). Sa description du raccourcissement de la ligne primitive nous semble cependant moins convaincante. L'auteur déclare que ce phénomène est beaucoup plus complexe que la régression et s'attarde un moment au fait remarquable, que le segment postérieur de la ligne paraît se raccourcir plus rapidement que le segment antérieur. Ce fait est attribué vaguement à une éventuelle condensation. La façon dont Spratt s'imagine le mécanisme du raccourcissement est dominée par sa conviction préconçue, que le segment postérieur participe au mouvement de régression que décrirait toute la ligne primitive. Le raccourcissement postérieur rapide est dès lors attribué à une dissolution: pendant que certaines parties de la ligne primitive s'invaginent encore pour former du mésoblaste, à l'extrémité postérieure d'autres parties s'étalent simplement en se perdant dans les feuillets extra-embryonnaires: ces parties 'move back into the *area opaca* during the growth of the embryo'. Alors que le mécanisme de la dissolution semble plausible, le mode de régression de la ligne primitive que Spratt propose, le semble moins. La structure très allongée de la ligne primitive, et son manque de rigidité, ne permettent en effet que difficilement de s'imaginer que, lorsqu'elle est refoulée à sa partie antérieure par la croissance du germe et la régression du nœud de Hensen, elle serait déportée globalement vers l'arrière, pendant que le segment postérieur se 'dissolverait'.

Le segment postérieur de la ligne primitive n'a suscité jusqu'à présent que peu d'intérêt. Les auteurs qui le décrivent ne le font souvent qu'incidemment et sans y revenir dans leur discussion. Il en est ainsi pour les segments postérieurs de

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jeunes blastodermes d'oiseaux sectionnés transversalement *in ovo* par Wetzel (1936). Waddington (1935) en a entrepris l'étude systématique: il s'agissait avant tout pour lui d'étudier jusqu'à quel niveau une section, pratiquée en arrière du nœud de Hensen, modifie le pouvoir de neuralisation dans ces fragments. Les expériences de Waddington ont été reprises de façon à peu près identique par Rudnick (1938), qui les a toutefois fait suivre par des mises en culture, soit *in vitro*, soit sur membrane chorio-allantoïdienne. Les résultats de ces deux auteurs, qui se rapportent à la capacité de neuruler, ont été confirmés et complétés par les travaux récents de Spratt. Quant aux mouvements qui peuvent se produire dans des fragments postérieurs isolés de lignes primitives, les opinions paraissent cependant différer.

La divergence se manifeste surtout dans la description du comportement du bord antérieur, donc de la ligne de section du fragment postérieur isolé. Selon Waddington et Rudnick des figures en V s'y dessinent régulièrement. Ils les attribuent à un mouvement de régression de la ligne primitive située en arrière du nœud de Hensen. Spratt discute la réalité de ces mouvements, lorsque le niveau de section est situé de 0,3 à 0,4 mm. en arrière du nœud. De plus, Waddington (1932, 1935), Wetzel (1936), mais surtout Rudnick (1938), ont constaté avec une certaine régularité des protrusions sur la ligne médiane, après culture de fragments postérieurs de lignes primitives. Dans des observations préliminaires, Spratt n'a au contraire vu ni régression, ni protrusions médianes. Dans sa discussion, il cite ces protrusions pour illustrer de prétendues contradictions dans les observations de ses prédécesseurs. Le but de nos expériences a dès lors été de contrôler les faits et si possible d'en concilier les interprétations afin d'en dégager la signification.

MATÉRIEL ET TECHNIQUES

Nous utilisons des œufs de poules Rhode Island rouges, que nous incubons à 38° C. pendant 19 à 21 heures. Après ce temps les germes atteignent des stades que nous désignerons comme 'ligne primitive allongée', 'prolongement céphalique court', 'prolongement céphalique long', 'repli céphalique jeune'. Les germes qui présentent d'autres stades après cette durée d'incubation n'ont pas été utilisés, quoique leur développement ne doive pas nécessairement être considéré comme anormal, vu la grande variabilité que présente la vitesse du développement des oiseaux au cours des premières heures d'incubation.

Dans une première série d'expériences, les observations de Spratt sur des blastodermes entiers aux différents stades cités ont été reprises. Nous avons utilisé ses techniques de culture (1951) et d'application de marques de charbon. Seize blastodermes ont été étudiés de cette façon.

Dans la majeure partie de nos expériences nous avons voulu soustraire la ligne primitive à l'influence de l'embryon en voie de formation. A cette fin nous avons sectionné les germes transversalement. Le plus souvent cette section

élimine le tiers antérieur de la ligne primitive; d'autres fois un peu plus que la moitié antérieure est éloigné. Le fragment postérieur est explanté après application de marques au charbon à des endroits, sur lesquels nous reviendrons en détail lors de l'exposé de nos résultats. Après la pose du fragment sur le milieu, l'excès de liquide est éliminé et des marques de référence sont appliquées sur le milieu même. Le germe ne se déplace généralement pas en entier par rapport à ces marques. Pendant les premières six heures, le déplacement des marques est suivi à la loupe, dessiné et mesuré. La durée de l'incubation des cultures est en règle générale de 15 heures. A ce moment, après de nouveaux dessins et de nouvelles mensurations les germes sont fixés le plus souvent, pour être coupés en série et colorés selon la technique de Unna. Quelques cultures sont incubées pendant 40 heures et traitées ensuite de la même façon. Nos résultats ont été obtenus par l'étude de 47 fragments postérieurs de blastodermes, qui portaient régulièrement 2 ou 3 marques.

OBSERVATIONS PERSONNELLES

A. *Germes entiers*

Nous voulons seulement citer brièvement ces premiers résultats: la régression du nœud de Hensen à 60–70 μ par heure, le raccourcissement de la ligne primitive, qui s'effectue au début le plus intensément dans le segment postérieur, l'invagination du mésoblaste, qui se poursuit après la formation de la ligne primitive et dont la vitesse atteint initialement 60 μ par heure pour diminuer progressivement. Ajoutons encore que la longueur de la ligne primitive, immédiatement avant l'apparition du prolongement céphalique, est de 1,50 à 1,75 mm. Toutes ces données correspondent à celles de Spratt, jusque dans les détails des vitesses de migration. Celles-ci sont légèrement plus basses, mais superposables en valeurs relatives.

B. *Deux tiers postérieurs de la ligne primitive*

1. *Dans les premières heures d'incubation*

Nous avons réparti nos germes en quatre groupes. Nous ne considérons comme normaux que les mouvements qui se produisent pendant les six premières heures après la mise en culture. De cette façon, nos observations se chevauchent largement, ce qui nous permettra de les intégrer facilement. Elles sont résumées sur le tableau 1.

(*a*) *Germes à ligne primitive allongée, immédiatement avant l'apparition du prolongement céphalique.* Une marque rostrale, c'est-à-dire appliquée en avant, sur la section de la ligne primitive, se déplace légèrement en direction caudale. La vitesse et l'étendue de ce mouvement sont trop irrégulières pour pouvoir être indiquées. Une marque latérale, située à côté de la ligne primitive, se déplace vers celle-ci avec une vitesse moyenne de 60 μ par heure, du moins à mi-hauteur de la ligne, où la zone qui exécute ce mouvement s'étend jusqu'à une distance de

300 μ de la ligne médiane. Parfois des marques de charbon participent à l'invagination cellulaire. On ne peut cependant pas attribuer une signification exagérée à la présence ou l'absence d'invagination des grains de charbon: les objections d'ordre technique sont trop nombreuses et trop fondées (cf. Spratt, 1947).

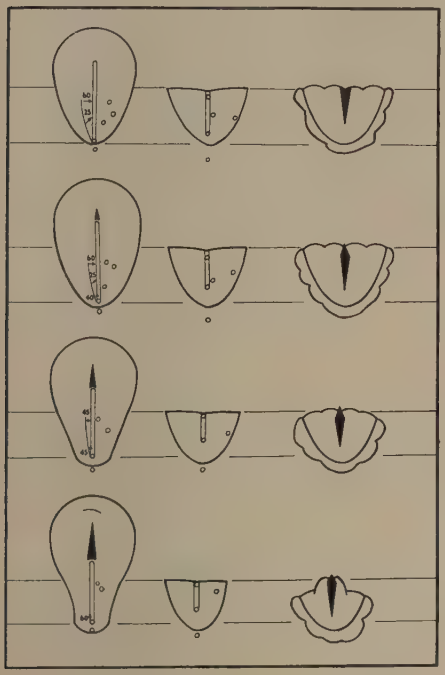

TABLEAU 1. Sur ce tableau sont schématisés: de haut en bas les stades utilisés; de gauche à droite l'évolution des fragments postérieurs isolés. La rangée de gauche montre les blastodermes entiers sur lesquels sont indiqués à droite l'emplacement de quelques marques typiques, à gauche la surface destinée à s'invaginer encore. Les flèches indiquent la direction des mouvements, les chiffres leur vitesse en microns par heure. La rangée du milieu montre entre les horizontales qui représentent les marques de référence, l'emplacement des marques figurées sur les schémas de gauche après 6 heures de culture. La rangée de droite montre les fragments postérieurs après 15 heures de culture.

Une marque latérale située en arrière du milieu de la ligne primitive n'atteint la ligne médiane que si elle se trouve plus près de celle-ci. La vitesse de migration est également plus basse. Il faut cependant remarquer que son déplacement est plus compliqué: elle est déportée en même temps vers l'avant. Le mouvement en cette direction, nul pour les marques situées à mi-hauteur de la ligne primitive, devient de plus en plus accusé lorsqu'on se rapproche de son extrémité postérieure. En intégrant ces mouvements, la vitesse absolue du déplacement des marques latérales est sensiblement égale.

Une marque posée sur l'extrémité postérieure de la ligne primitive peut encore, au cours des deux premières heures, se déplacer légèrement en direction postérieure. Le sens du mouvement est cependant brusquement renversé. La vitesse moyenne de ce mouvement en direction antérieure, calculée après les six premières heures d'incubation, est de 20 μ par heure. Cette valeur peu élevée s'explique par le fait qu'il s'agit en réalité de la résultante des deux mouvements

en sens opposés que nous venons de décrire. Sous la réserve que nous venons de formuler, nous pouvons signaler n'avoir jamais constaté l'invagination de marques posées à l'extrémité postérieure de la ligne primitive.

Des marques situées en arrière de l'extrémité postérieure de la ligne primitive ou en dehors de l'aire d'invagination (cf. tableau 1) migrent en direction postérieure ou latérale avec une vitesse de 40–50 μ par heure.

(*b*) *Germes à prolongement céphalique court.* Il nous a semblé qu'à ce stade la ligne primitive atteint sa longueur maximum (1,70 mm. en moyenne). Des marques rostrales peuvent encore se déplacer en direction postérieure. Ce mouvement est encore moins régulier que sur des germes plus jeunes. Des marques latérales, situées à une distance maximum de 200 μ du milieu de la ligne primitive, peuvent encore migrer jusqu'à la ligne médiane. Les marques placées plus en arrière combinent de nouveau deux mouvements, alors que des marques réellement postérieures se dirigent exclusivement vers l'avant à une vitesse moyenne de 40 μ par heure.

(*c*) *Germes à prolongement céphalique allongé.* Des marques rostrales ne se déplacent pratiquement plus ou esquissent un très faible mouvement en direction postérieure. Des marques latérales peuvent encore atteindre la ligne primitive lorsqu'elles n'en sont pas plus éloignées que de 150 μ. L'aire d'invagination est devenue encore plus étroite. Des marques postérieures avancent avec une vitesse de 45 μ par heure. Comme à tous les autres stades étudiés, nous retrouvons dans l'ectoblaste, qui entoure la zone d'invagination, un mouvement divergent, qui atteint la vitesse absolue de 40–50 μ par heure. Cette vitesse est du même ordre de grandeur que celle du mouvement convergent.

(*d*) *Germes à repli céphalique.* Les marques rostrales demeurent immobiles. Après six heures, elles sont parfois dépassées en profondeur par du matériel cellulaire qui migre vers l'avant sur la ligne médiane et immédiatement à côté de celle-ci. L'aire d'invagination ne s'étend plus qu'à 50 μ au maximum de part et d'autre de la ligne primitive. L'extrémité postérieure de celle-ci progresse rapidement: 60 μ par heure. Le matériel cellulaire qui entoure la partie postérieure de la ligne s'éloigne de façon concentrique à une vitesse absolue d'environ 50 μ par heure.

2. *Après 15 heures d'incubation*

Après ce temps d'incubation, les données sont moins uniformes et ne peuvent plus être traitées quantitativement. Une tendance générale se dégage cependant des résultats obtenus. Les explants réalisés aux divers stades se rapprochent tous d'un même type, que nous schématisons par stade sur le tableau 1. Les points essentiels se situent au niveau du milieu de la ligne de section. Le stade 'ligne primitive allongée' et le stade 'repli céphalique' pourront suffir à la description, les autres présentent des résultats intermédiaires. Dans les fragments les plus jeunes la partie médiane antérieure reste le plus souvent apparemment rétractée.

Parfois il s'agit d'une régression réelle; parfois cette rétraction n'est que suggérée par la progression des ailes mésoblastiques latérales. Les germes les plus âgés présentent typiquement un tout autre aspect: sur la ligne médiane s'est formée une protrusion blastématique projetée régulièrement en avant du niveau de section.

Au microscope ces protrusions apparaissent comme de véritables blastèmes très basophiles. Du côté ectoblastique on retrouve en surface des restes de la ligne primitive; ils sont plus ou moins en continuité avec le blastème situé en profondeur (Planche, fig. 1). Ce matériel profond est plus ou moins massif selon les cas. Parfois on y reconnaît des dispositions qui rappellent les organes axiaux: du mésoblaste axial et peut-être aussi de la chorde dorsale (Planche, fig. 2). A part l'absence de tissu nerveux, la similitude de ces images avec celles obtenues par des coupes de 'queues' produites par des fragments antérieurs contenant le nœud de Hensen, est frappante. Dans l'ectoblaste derrière le blastème, où on chercherait normalement la moelle épinière, les indices de neuralisation sont ou bien totalement absents ou extrêmement discrets. Ceci confirme les observations de tous nos prédécesseurs.

3. *Après 40 heures d'incubation*

En ce qui concerne les données quantitatives, la même remarque s'impose qu'après 15 heures d'incubation. Nous retrouvons cependant de façon remarquablement constante, dans la partie antérieure du fragment explanté, un blastème qui peut être entouré de structures rappelant un sinus rhomboïde. Au microscope ce blastème se présente comme une élévation, éventuellement entourée de replis épiblastiques (Planche, fig. 3, 4). Plus rarement qu'après 15 heures d'incubation, on trouve une traînée cellulaire axiale. Ici également la question se pose si on peut l'assimiler à de la chorde dorsale ou une structure homologue.

C. *Deux cinquièmes postérieurs de la ligne primitive*

Aux différents stades, le comportement des marques latérales et postérieures est identique à celui des marques posées à des endroits correspondants de fragments comprenant $\frac{2}{3}$ de la ligne primitive. Les marques rostrales cependant se comportent de façon différente. Probablement à cause de leur position plus reculée, aucun mouvement de régression n'a pu être décelé au cours des premières heures. D'autre part, déjà après 4 à 6 heures, les marques rostrales sont dépassées en profondeur par une poussée de cellules dirigée vers l'avant, et qui déborde la ligne de section. Il s'agit probablement de matériel récemment invaginé qui se porte en avant sur la ligne médiane. Il est remarquable que ceci peut s'observer déjà après 6 heures d'incubation et même sur les germes les plus jeunes de notre série.

DISCUSSION

1. *Régression et raccourcissement de la ligne primitive*

Quand on compare nos observations à celles de Spratt, il peut paraître à première vue étonnant que nous proposons une interprétation différente des mouvements à l'intérieur de la ligne primitive et du mécanisme de son raccour-

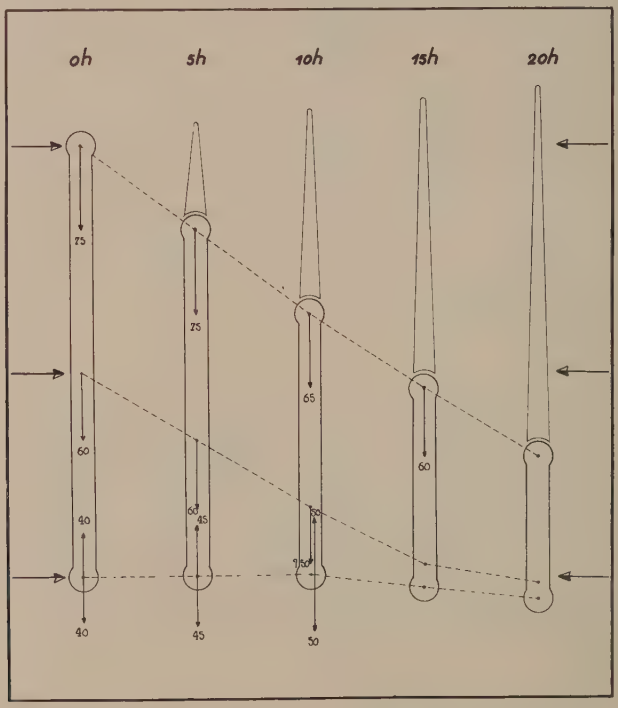

TABLEAU 2. Sur ce tableau nous reproduisons sous forme de vecteurs les vitesses de migration (indiquées en micron/heure) que nous avons observées dans la ligne primitive. A partir du stade de la ligne primitive pleinement développée nous avons calculé sur des stades choisis arbitrairement toutes les 5 heures les transformations et déplacements que subirait la ligne primitive si les mouvements que nous avons pu retrouver étaient intégrés (cf. texte). Cette reconstruction théorique fournit des résultats qui se rapprochent presque parfaitement des observations faites par Spratt et que nous avons pu confirmer.

cissement. La différence essentielle de conception réside dans le fait que nous essayons de voir les différents mouvements dont le blastoderme est le siège comme autant de constituants intégrés des phénomènes de croissance et de morphogénèse.

On peut distinguer dans le blastoderme de poulet trois grands mouvements

morphogénétiques intégrés (tableau 2). A la périphérie de l'*area pellucida*, du moins dans la moitié postérieure, un large mouvement épibolique étale les feuillets extra-embryonnaires à une vitesse d'environ 40 μ par heure. Ce mouvement centrifuge ne possède pas de centre réel parce que le germe ne possède plus une forme concentrique au moment où nous l'étudions. Nous devons situer plus ou moins approximativement le point zéro des mouvements épiboliques. Il nous semble pouvoir être localisé sans risque exagéré à la base du jeune prolongement céphalique. Dans le neurectoblaste qui est presqu'entièrement isolé dans le fragment antérieur du blastoderme par une section au niveau du tiers antérieur de la ligne primitive, les mouvements morphogénétiques de croissance et de neuralisation s'effectuent de telle façon que notre point zéro mérite assez bien son nom.

Les phénomènes morphogénétiques de la neuralisation auxquels nous venons de faire allusion sont évidemment en corrélation avec un autre mouvement de très grande importance. Il s'agit de la régression du nœud de Hensen. Leur interdépendance n'est pas absolue: le nœud de Hensen possède des facultés de régression même lorsqu'il n'est plus 'poussé' par le système nerveux. Mais ce problème ne nous intéresse pas spécialement ici: nous tenons surtout à faire remarquer que la tendance à la régression présente son maximum au niveau du nœud, mais ne fait pas entièrement défaut dans la partie antérieure de la ligne primitive.

L'étude du phénomène de raccourcissement de la ligne primitive qui ne nous semblait pas entièrement élucidé par les recherches de Spratt, nous a amené à mettre en évidence un mouvement jusque maintenant méconnu. Il s'agit du mouvement en direction antérieure de l'extrémité postérieure de la ligne primitive. Les deux mouvements, en directions opposées, à l'intérieur de la ligne primitive, sont évidemment responsables du raccourcissement de la ligne primitive. Seule l'expérience d'isolement que nous avons tentée pouvait permettre de les distinguer et d'affirmer ainsi leur importance égale dans le mécanisme du raccourcissement.

Regardons maintenant de plus près ces mouvements, observables artificiellement, et essayons de les intégrer. Les faits connus sont: la régression du nœud de Hensen à une vitesse d'environ 75 μ par heure par rapport au point zéro convenu et l'épibolie qui au niveau de la ligne primitive est également dirigée vers l'arrière, à une vitesse d'environ 40 μ par heure. Il semble très plausible d'admettre avec Spratt que la ligne primitive dans son ensemble située entre le nœud de Hensen et un point immédiatement en arrière de son extrémité postérieure, participe au mouvement de régression. Ce mouvement de régression n'est cependant pas égal sur toute la longueur de la ligne primitive: une marque placée au milieu de la ligne primitive d'un blastoderme entier régresse à une vitesse d'environ 55–60 μ par heure. Quand nous portons comme vecteurs sur le tableau 2 les trois vitesses de régression observées, nous distinguons mieux encore leur chute à partir du nœud. Maximum au nœud, elle est intermédiaire

au niveau de la partie moyenne de la ligne, et minimum immédiatement en arrière de son extrémité postérieure. La vitesse de régression diminue de 75 μ à 60 et 45 μ par heure. Par cette chute des vitesses seule, la ligne primitive se raccourcirait. La vitesse de ce raccourcissement ne serait toutefois que de 30 μ par heure, ce qui ne correspond pas à la réalité. Remarquons cependant que cette valeur correspond exactement à la vitesse de raccourcissement de la moitié antérieure de la ligne primitive qui, au stade du prolongement céphalique court, est de 15 μ par heure.

Dans la partie postérieure de la ligne primitive, cependant, le raccourcissement s'effectue à une vitesse beaucoup plus grande. Cette différence s'explique par les mouvements que nous y avons révélés. Après section, cette partie postérieure, devenue capable d'extérioriser ses tendances autonomes, effectue des mouvements en sens antérieur. La vitesse de ces mouvements est de 40–60 μ par heure. De l'intégration de ce mouvement avec celui de la régression résulte un arrêt, du moins pendant les premières heures d'incubation. C'est ce que l'on voit lors d'observations sur blastodermes *in toto*. Ce n'est que plus tard que l'influence de la régression devient prépondérante et fait effectuer un léger recul à l'extrémité postérieure de la ligne primitive. Aussi longtemps que ce pôle postérieur reste apparemment immobile, la vitesse de raccourcissement de la ligne reste du même ordre de grandeur que celle de la régression. Comme nous venons de l'indiquer, cette concordance n'est cependant qu'accidentelle: elle est due au fait que le raccourcissement autonome de la partie postérieure de la ligne primitive est du moins au début compensé par la vitesse de la régression de son extrémité postérieure.

La description que nous venons de donner de la régression et du raccourcissement de la ligne primitive, ne contredit en aucun point les données que Spratt (1947, p. 75) a résumées dans son tableau devenu classique. Nous croyons avoir apporté des précisions quant au mécanisme intime selon lequel s'effectuent ces mouvements.

Notre conception nous semble jeter une lumière nouvelle sur les mouvements que l'on observe à la ligne de section d'un fragment postérieur de ligne primitive. On a décrit jusqu'à présent ou bien des figures en V, que nous avons retrouvées, ou bien des protrusions sur la ligne médiane pour lesquelles toute tentative d'explication manque. La formation de ces blastèmes, décrits par Waddington et par Rudnick, ne constitue pas une contradiction comme semble le suggérer Spratt, avec une déformation en V légère et initiale de la ligne de section. Au contraire, notre conception permettrait de les prévoir. Des recherches systématiques nous ont en effet appris qu'il s'agit ici d'une impulsion de croissance qui part dans la partie postérieure de la ligne primitive et qui se prolonge en avant lorsqu'elle est libérée de ses entraves comme c'est le cas ici. L'observation de ces mouvements antérieur et postérieur tend en outre à faire accepter qu'un point situé près du milieu de la ligne primitive pourrait être le centre vers lequel convergent les mouvements autonomes dans la ligne.

2. *Nature du matériel mésoblastique s'invaginant par la partie postérieure de la ligne primitive*

Spratt (1947) a démontré la présence de matériel mésoblastique situé en surface au stade de la ligne primitive tardive. Nous pouvons confirmer ses résultats. Les vitesses de migration que nous avons trouvées concordent d'ailleurs parfaitement avec celles de Spratt, du moins sur des blastodermes *in toto*. Sur des fragments qui ne contiennent que les $\frac{2}{3}$ postérieurs, nous avons trouvé des mouvements d'invagination en directions légèrement différentes. Ceci se rapporte surtout au mouvement de progression du pôle postérieur de la ligne que nous oserions appeler 'nœud postérieur'. Initialement nous ne trouvons cette progression que dans la moitié postérieure de la ligne primitive. Après une durée d'incubation plus grande cette impulsion peut se continuer jusqu'en avant de la ligne de section du fragment.

Les résultats obtenus après 15 heures et plus nettement encore après 40 heures d'incubation, tendent à démontrer qu'il s'agit ici d'une partie importante du matériel destiné à former la queue. L'aspect à la loupe aussi bien qu'au microscope démontre que du mésoblaste de queue s'est amassé ici. Les mouvements cellulaires qui en sont responsables sont une combinaison de mouvements d'invagination et un mouvement antérieur. Cette progression conduit à la fermeture sans invagination médiane de la partie postérieure de la ligne primitive, dans laquelle le 'nœud postérieur' se comporte à la façon d'un bouton de fermeture-éclair.

Ces mouvements complexes d'invagination-progression du mésoblaste, combinés au raccourcissement par une forme de concrescence de la partie postérieure de la ligne primitive, nous mènent aux structures reconnaissables après 15 heures de culture. Nous trouvons alors un mésoblastème dans lequel des organes axiaux sont nettement reconnaissables. Après 40 heures il devient parfois plus difficile de retrouver des organes axiaux, mais un blastème s'est alors formé dans lequel on reconnaît un bourgeon caudal, parfois entouré par des structures rappelant un sinus rhomboïdal.

Quelle demeure alors la contribution du nœud de Hensen à la formation du bourgeon caudal? Les recherches approfondies de Spratt ont confirmé la notion de la nécessité du nœud de Hensen pour la formation de la chorde. Beaucoup d'autres auteurs avant et après lui sont d'ailleurs arrivés à la même conclusion. Ceci nous permet de nous attendre en principe à ce que le nœud soit indispensable également à la formation de la queue à partir du bourgeon caudal. D'autre part nous n'avons jusqu'à présent pu démontrer la présence de chorde organisée dans les petits bourgeons caudaux qui se forment dans des fragments postérieurs de blastodermes. L'interprétation suivante nous paraît provisoirement la meilleure: chez les embryons de poulet la queue se formerait à partir de matériel qui s'invagine tardivement par la partie postérieure de la ligne primitive. Il est très probable que le matériel destiné à former la chorde caudale s'y

trouve également. L'élévation et l'élongation du bourgeon caudal pourraient résulter de la convergence des deux courants opposés qui se rencontrent environ au milieu de la ligne primitive: d'un côté le courant qui part du nœud postérieur amenant le matériel cellulaire, de l'autre le courant qui part du nœud de Hensen et qui imprime au bourgeon caudal sa destinée définitive. Il est trop tôt pour traduire définitivement les phénomènes dans la terminologie de Spratt. La disposition des matériels présomptifs est toutefois très comparable à celle que l'on trouve dans le segment antérieur de la ligne primitive. Elle est seulement masquée par leur rassemblement en un bourgeon avant que le 'chorda-bulb' ne l'atteigne. Selon l'hypothèse de Spratt, l'impulsion 'organisatrice' du 'chorda-bulb' serait indispensable à la formation d'une queue. Malgré les belles analyses de Spratt, ce mode de développement semble occuper une place tellement spéciale dans le développement des Vertébrés, qu'on hésite de l'accepter avant de l'avoir mis à l'épreuve expérimentalement.

3. *Critique de la méthode et contribution de la régulation aux résultats obtenus*

On trouve une critique approfondie de la méthode aux marques de charbon dans l'article de Spratt (1947). L'auteur y omet seulement d'insister suffisamment sur les désavantages de la méthode de culture, à laquelle vont ses préférences. Comme l'a fait remarquer Pasteels (1953), son défaut principal est son influence freinatrice sur les mouvements morphogénétiques. Dans nos expériences, nous croyons y échapper dans la mesure du possible parce que nous suivons le raccourcissement de la ligne primitive à des stades différents très rapprochés, et cela seulement pendant une période relativement brève.

La concordance de nos résultats sur germes *in toto* avec ceux de Spratt ne fait aucun doute. Dans ce cas ils sont parfaitement comparables, jusque dans les détails des vitesses de migration. Les valeurs légèrement plus basses que nous trouvons peuvent être attribuées à des différences de matériel et peut-être de température d'incubation. De plus, après section du blastoderme, la progression de la partie postérieure de la ligne primitive ne semble pas due à une lésion traumatique puisque les vitesses d'immigration du mésoblaste à ce niveau sont les mêmes que celles que l'on constate sur des germes *in toto*.

Une fois admise l'exactitude de nos observations, il reste toujours possible d'en discuter la signification réelle, en objectant que des mouvements anormaux se produisent peut-être sous l'influence de la régulation. Il faut admettre qu'il s'agit là d'un argument théorique irréfutable. D'autre part il y a lieu de remarquer que les expériences en général et celles-ci en particulier visent précisément à faire fournir par les germes des prestations qu'ils ne réalisent pas normalement, et que seules les conditions expérimentales permettent. Généralement on ne reconnaît ces tendances dans le développement normal que lorsque l'expérience y a attiré l'attention.

Nous voulons dès lors examiner brièvement si l'urogénèse du Poulet telle

qu'elle est présentée ici peut éclaircir des données bien établies du développement.

En premier lieu s'explique ainsi facilement la difficulté déjà entrevue, qui surgit quand on accepte la conception de Spratt sur le raccourcissement de la ligne primitive sous l'influence d'une poussée antérieure.

En second lieu, l'extension de la ligne primitive: selon Spratt la ligne primitive, au stade du prolongement céphalique long, se situerait plus loin en arrière dans l'*area opaca* qu'aux stades immédiatement plus jeunes. Nos observations sur une centaine de blastodermes, spécialement examinés à ce point de vue, nous ont permis de constater des faits légèrement différents. Après l'apparition du prolongement céphalique la ligne primitive empiète de moins en moins sur l'*area opaca*. Cela peut évidemment s'expliquer par une épibolie rapide de l'aire extra-embryonnaire, mais ne plaide aucunement contre le mécanisme de raccourcissement de la ligne primitive que nous proposons.

Il y a enfin le développement normal du bourgeon caudal. Ce bourgeon se forme après 45 heures d'incubation environ et est situé alors en avant des vestiges de la ligne primitive. Ceux-ci restent reconnaissables malgré la formation du bourgeon caudal, qui se dessine en outre avant que la tige chordale n'atteigne son niveau. La formation autonome du bourgeon caudal, en avant de l'extrémité postérieure de la ligne primitive, peut s'expliquer facilement par notre interprétation.

Cette série d'arguments ne fournit pas encore une démonstration suffisante, mais permet cependant de supposer avec une forte probabilité que les mouvements morphogénétiques menant à la condensation du matériel du bourgeon caudal correspondent à des mouvements qui, dans le germe intact, se compensent et par cela se cachent partiellement.

La discussion de l'analogie éventuelle de l'urogénèse des Amphibiens et des Oiseaux ne pourra être faite qu'après vérification de la présence de matériel chordal dans des fragments postérieurs isolés. Ce n'est que dans ce cas qu'on pourra parler d'analogie complète.

RÉSUMÉ

1. Des fragments comprenant les $\frac{2}{3}$ ou $\frac{2}{5}$ postérieurs de la ligne primitive à partir de son plein développement jusqu'au stade du repli céphalique ont été cultivés *in vitro* après application de marques de charbon.

2. Dans ces conditions l'extrémité postérieure de la ligne primitive extériorise nettement un mouvement en direction antérieure, qui peut mener jusqu'à la formation d'une protrusion médiane antérieure après 15 heures de culture et à l'élévation d'un bourgeon caudal après 40 heures.

3. La révélation de ce mouvement, masqué dans le blastoderme entier par les mouvements d'épibolie et de régression, permet de comprendre la façon dont se produit le raccourcissement initial de la ligne primitive dans la moitié postérieure.

SUMMARY

1. Fragments of chick blastoderm containing the posterior two-thirds or two-fifths of the primitive streak have been cultured, using Spratt's explantation and carbon particle marking techniques. The stage of embryo at operation ranged from full primitive streak to headfold.

2. Under these circumstances a forward movement was observed in the posterior half of the primitive streak, giving rise after 15 hours of cultivation to an anterior median protrusion which contains structures plainly derived from the primitive streak (fig. 1) or even a distribution of cell-strands recalling axial organs (fig. 2). After 40 hours of cultivation a tail-bud may arise, obviously without any contribution from the node of Hensen.

3. We therefore suggest that the shortening of the streak should not be considered merely as a regression but as the consequence of three simultaneous and integrated movements: (*a*) regression of the node and anterior half of the streak; (*b*) a general epibolic movement of the whole blastoderm; (*c*) a forward movement of the posterior part of the streak, most marked at the posterior end, for which the name of 'posterior node' is proposed.

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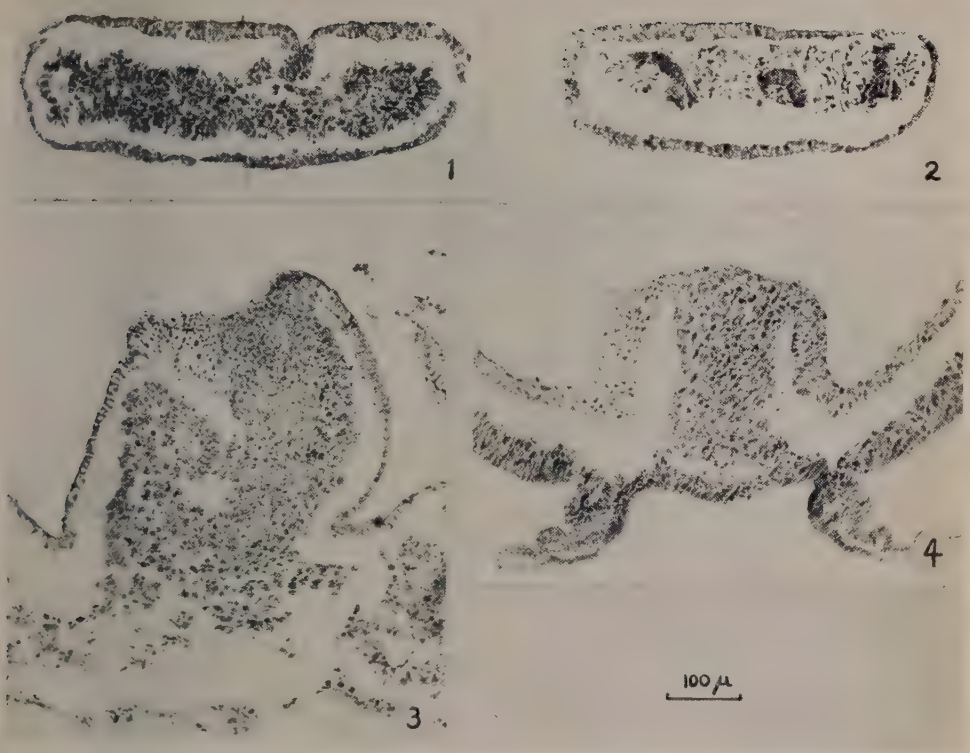
EXPLICATION DE LA PLANCHE

Fig. 1. Coupe transversale d'une protrusion au milieu de la ligne de section d'un fragment ($\frac{3}{5}$) postérieur d'une ligne primitive complètement allongée, après 18 heures de culture. Image d'une ligne primitive et de mésoblaste latéral. Absence de tissu nerveux. (Coloration Unna–Brachet.)

Fig. 2. Coupe transversale d'une protrusion médiane en avant de la ligne de section d'un fragment ($\frac{2}{5}$) postérieur d'une ligne primitive complètement allongée, après 18 heures de culture. Image rappelant la disposition d'une chorde flanquée de deux rangées de somites. Absence de tissu nerveux. (Coloration Unna–Brachet.)



L. VAKAET

FIG. 3. Coupe transversale d'une élévation apparue sur un fragment ($\frac{2}{3}$) postérieur d'une ligne primitive avec prolongement céphalique jeune après 40 heures de culture. Aspect rappelant les images typiques d'un bourgeon caudal. (Coloration Unna–Brachet.)

FIG. 4. Bourgeon caudal typique (embryon après 48 heures d'incubation). (Coloration Unna–Brachet.)

(Manuscript received 12:vi:59)

Experimental Neoplastic Formation in Embryonic Chick Brains

by BENGT KÄLLÉN[1]

From the Tornblad Institute of Comparative Embryology, Lund

WITH TWO PLATES

In mammalian teratology, a malformation consisting of 'overgrowth' of the neural tube has been described by, among others, Patten (1952, 1957) and Ariëns Kappers (1956, 1957). Sjodin (1957) thought it to be a post-mortem effect and not a true malformation. The present author (1955) demonstrated that a similar malformation could be produced in the rostral part of the brain of chick embryos by operations at somite stages on the rostral end of the rhombencephalon which damaged underlying notochordal structures. Bergquist (1959 *a*, *b*) has discussed this problem further. The overgrowth is made up of an excessive proliferation in the neural epithelium with a reduced cell differentiation, giving rise to only a thin layer of migrated cells but to a marked development of neural epithelium in 4- to 5-day embryos. The vesicles of the hemispheres and mesencephalon are strongly folded, and Bergquist (1959*c*) has produced evidence that the folding is due to the accumulation of mitoses along the ventricular lining of the vesicles. For further details of this malformation the reader is referred to Bergquist's papers.

In the present paper data are presented which provide evidence for a neoplastic development in the overgrown brain parts.

MATERIAL AND METHODS

Chick embryos at stages between 8 and 18 somites were vitally stained with neutral red, and, after removal of the vitelline membrane, the rhombomere nearest to the rostral part of the brain was extirpated with the aid of glass needles. The eggs were then sealed and incubated. The embryos were killed in Bouin's fluid at ages varying between 3 and 12 days of incubation. All 16 embryos fixed were living at the time of fixation. Serial sections at 10 or 15 μ were prepared and stained with haematoxylin and eosin.

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RESULTS

Overgrowth was obtained in nearly all the embryos operated on in this way. The mortality rate was, however, about 60 per cent.

As was stressed by Bergquist, very different degrees of overgrowth may occur in different individuals similarly operated. In rare cases no disturbances could be found macroscopically, and only after examination of the sections could abnormalities indicating overgrowth be seen. In one embryo, for instance, the only abnormality is a folding of the mesencephalon on one side. Usually, however, the disturbance of growth could easily be seen externally and was pronounced in sections. In some cases very localized though well-developed abnormalities were present. In Plate 1, fig. A a hemisphere with such a localized malformation is shown, and sometimes similar conditions could also be found in the mesencephalon.

Bergquist (1959*a*) has given a detailed description of the changes in morphology in embryos aged 4 to 5 days, and his observations were verified in the present material. A fairly regular neural epithelium may be observed at this stage, abnormal only in its strong folding and high mitotic activity. The present author has, however, also observed numerous mitoses among the lateral cells in the neural epithelium (Plate 1, fig. B), a feature rarely found in normal embryos. Rosette formations in the epithelium as described by Bergquist (1959*a*) and found in cases of spontaneous overgrowth (Ariëns Kappers, 1956, 1957) were also seen.

In older stages, very different pictures could be seen. In some cases a fairly normal histogenetic development of the brain-wall seems to have taken place, to judge from the haematoxylin-eosin stained slides. An example has already been mentioned and another is given in Plate 1, fig. C, taken from an embryo with a pronounced abnormality of the mesencephalon. The histological picture of the highly malformed brain part is relatively normal. Fibres developing from such tissue are often seen to grow atypically, sometimes forming aberrant nerves which leave the brain surface at abnormal sites.

In many cases, however, the later growth of the 'overgrown' tissue is apparently unorganized. In Plate 2, fig. A the mesencephalon of one embryo is shown, and here the normal morphology is completely lost and replaced by a fairly compact structure. A similar appearance was found in other embryos. The histology of this tissue is very variable. In some parts (Plate 1, fig. D) a fairly normal histology is found with regular strands of cells and fascicles. In other parts. the tumour formation consists of a dense mass of small and uniform cells with few or no signs of differentiation (Plate 2, fig. B). The cells had a tendency to become arranged in rosette formations in many places.

A tendency of the cells to break through the external brain surface and infiltrate into the surrounding mesenchyme could sometimes be seen in embryos with a highly malformed brain (Plate 2, fig. C).

DISCUSSION

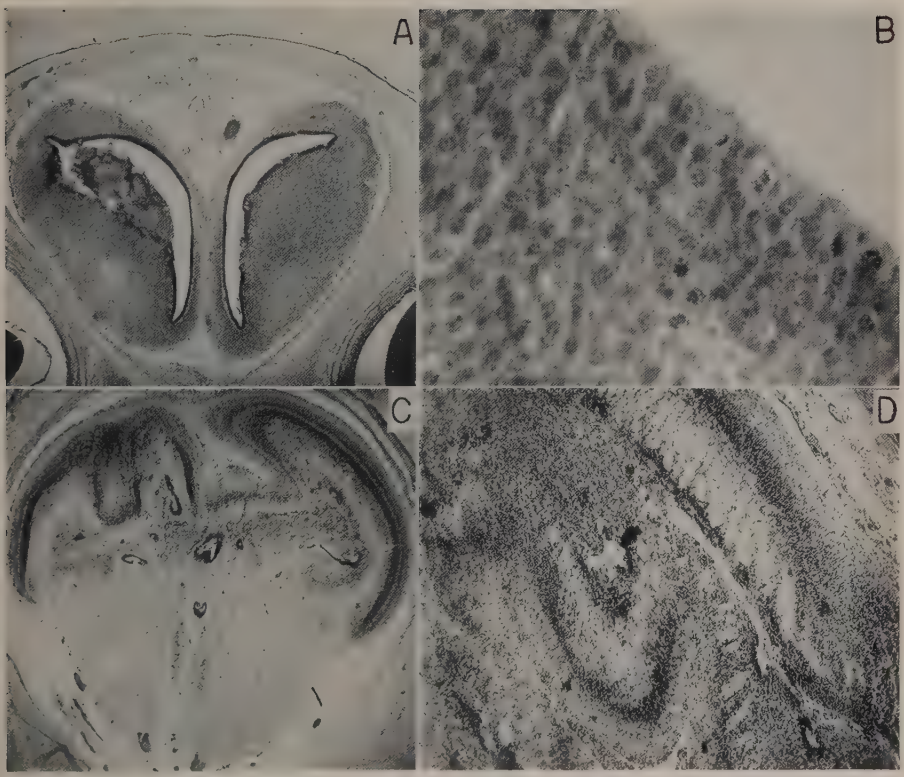
The disturbance which causes the 'overgrowth' phenomenon in the present experiments is undoubtedly due to an increased mitotic activity of the neural epithelium, giving rise to the folding and to the poor differentiation of the brain-wall found in young stages. The degree of disturbance and the localization of the overgrowth phenomenon seems to vary considerably in different embryos for reasons which are not yet known. Similarly, the further development of the 'overgrowth' tissue may occur in different ways in different embryos. Sometimes a fairly normal histogenesis of the cells takes place, giving rise to a brain-wall which may be morphologically abnormal but which contains differentiated cell elements. In most cases, in parts of the 'overgrowth', neoplastic-like formations develop with unorganized growth of poorly differentiated cells, which are small, densely packed, and continue mitotic division at a rate which is unknown in normal development for that age.

Cases of invasive growth of the tumour cells have been observed, but it is not clear whether this is an expression of malignancy. During normal development the neural crest cells show the same ability to invade surrounding mesenchyme, and the infiltration observed in 'overgrowth' cells might well be such a change in cell potency and not a true malignant degeneration.

It is, of course, difficult or impossible to try and draw any exact parallels between the neoplastic formations obtained from 'overgrowth' embryos, and similar phenomena from human pathology. The closest parallel would be tumours belonging to the medulloblastoma group. These tumours are supposed to develop from the embryonic granular layer of the cerebellum, a tissue which from a physiological point of view is comparable to the neural epithelium under discussion in the present paper. Some histological features of the 'overgrowth' neoplasms also agree with those of medulloblastoma, such as the tendency towards rosette formation. In other cases, where the cell picture is less anaplastic, similarities to ependymoma tumours can also be seen. The phenomenon of 'overgrowth' may possibly be etiologically related to these tumours.

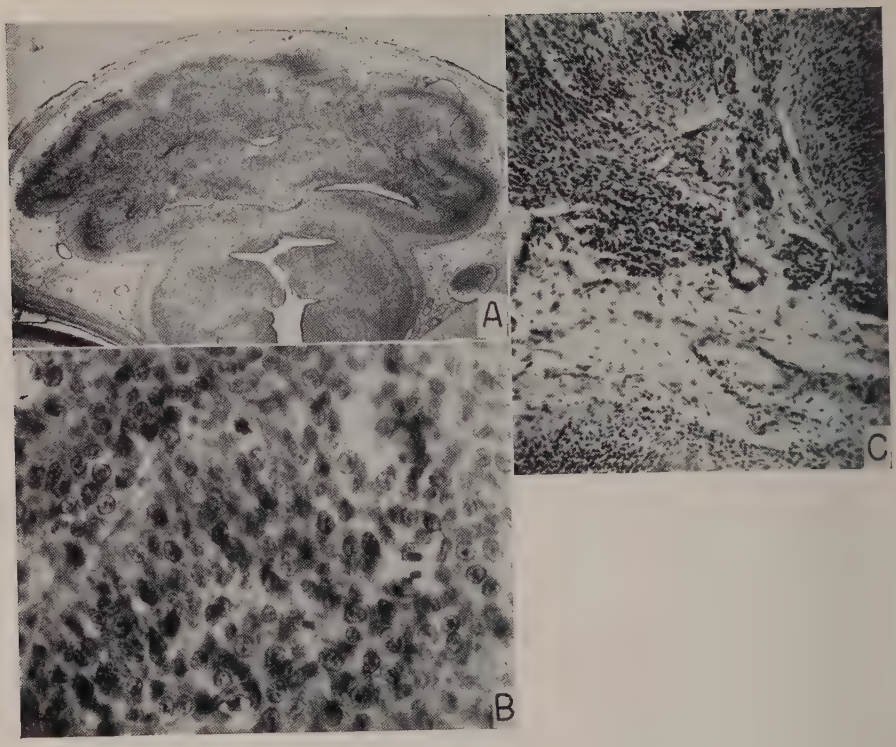
SUMMARY

After operations on somite stages of chick embryos in the rostral part of the rhombencephalon, a malformation of the rostral part of the brain develops, consisting of so-called overgrowth. The neural epithelium proliferates actively and gives rise to a strong folding of the brain-wall. During later development of such embryos, the overgrown tissue may differentiate in a fairly normal way or may develop into neoplastic formations, consisting of small, densely packed cells. Mitoses occur in such cells. There is a tendency towards rosette formation. Cases of invasive growth into the surrounding mesenchyme have been found. The neoplastic formations are compared to human medulloblastoma tumours.



B. KÄLLÉN

Plate 1



B. KÄLLÉN

Plate 2

ACKNOWLEDGEMENT

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EXPLANATION OF PLATES

Plate 1

Fig. A. Transverse section of the hemisphere of embryo SA, 7d, 23 hours. Note localized tumour formation on left. Haematoxylin-eosin, 10 μ. ×15.

Fig. B. Detail of neural epithelium of the overgrown mesencephalon of embryo S6, 4d, 22 hours. A group of mitoses among lateral neural epithelium cells can be seen. Haematoxylin-eosin, 10 μ. ×590.

Fig. C. Transverse section of overgrown part of the mesencephalon of embryo S159, 7d, 23 hours. Note fairly normal histogenesis in the malformed brain. Haematoxylin-eosin, 10 μ. ×17.

Fig. D. Transverse section through part of the overgrown mesencephalon of embryo S50, 9d, 6 hours. The histological differentiation in this region is relatively normal. Haematoxylin-eosin, 15 μ. ×60.

Plate 2

Fig. A. Transverse section through part of the overgrown mesencephalon of embryo SA, 7d, 23 hours, showing tumorous malformation in the *tectum opticum*. Haematoxylin-eosin, 10 μ. ×16.

Fig. B. Transverse section through part of the overgrown mesencephalon of embryo SA, 7d, 23 hours, showing tumour cells. Note uniform cell picture of immature nuclei. Mitoses can be seen. Haematoxylin-eosin, 10 μ. ×590.

Fig. C. Transverse section of the mesencephalon of embryo S50, 9d, 6 hours. Cells have grown from the tumour tissue into the surrounding mesenchyme. Haematoxylin-eosin, 15 μ. ×140.

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The Occurrence and Morphogenesis of Melanocytes in the Connective Tissues of the PET/MCV Mouse Strain[1]

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WITH ONE PLATE

INTRODUCTION

MAMMALS, as a rule, are described as having melanocytes of neural crest origin confined almost entirely to the skin. Of the organs other than skin which have been described as possessing melanocytes are portions of the gonado-genital apparatus of the Opossum (Burns, 1939), and, in the house mouse, tissues of the nictitans, the meninges of the brain, the parathyroids, the thymus and harderian glands (Markert & Silvers, 1956), and the parathyroids of C58 mice (Dunn, 1949).

The present investigation has been made in a strain of mice in which melanocytes are found in the connective tissues throughout much of the body. This strain originated several years ago in the Department of Genetics, Medical College of Virginia, from a cross between inbred C_3H and black mice of unknown breed obtained from a local pet shop. Because of the latter circumstance, the line-bred progeny have been termed the PET/MCV strain. Upon the discovery by the authors (Reams & Nichols, 1959) of melanocytes in the serosae of new-born PET/MCV mice, a stock was developed for investigation.

The presence of melanocytes in the coelomic lining of the chick and other fowl has been known for years (Willier & Yuh, 1928; Eastlick, 1939; and Rawles, 1940, 1945). Recently, Reams (1956) has shown that peritoneal pigment cells are excellent subjects for the study of the ontogeny of cell form since they are easily obtained for experimentation, and since the changes which they undergo in their morphogenesis are readily apparent due to the onset of melanogenesis prior to the development of their definitive shape. The melanocytes within the connective tissues of the PET/MCV mouse similarly lend themselves to analysis by experimental techniques.

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It is the purpose of this paper to describe the distribution of melanocytes in the connective tissues of the PET/MCV mouse and to present experimental data on the morphogenesis of these pigment cells.

MATERIALS AND METHODS

In order to survey the progressive appearance and morphology of melanocytes throughout the body of the PET/MCV strain, individuals ranging in age from 14 days prenatal to adults were killed and fixed in Tellyesniczky's formol-aceto-alcohol. The mice were then hemisected longitudinally, dehydrated in alcohol, cleared in oil of wintergreen, and examined for the presence of melanocytes. Five or more mice from different litters of each group were examined.

A technique of intracoelomic grafting similar to that described by Rawles (1947) was employed to investigate the developmental capacity of melanocytes from the serosae. In addition, grafts were made to the skin of host chick embryos by the method of Willier, Rawles, & Hadorn (1937). In all cases the donor tissue (0·2 mm.^{3} fragments of serosa bearing spherical melanocytes) was taken from PET/MCV mice varying in age from 18-day prenatal to 4-day postnatal and grafted into 60–70-hour White Leghorn chick embryo hosts. Following the grafting of mouse tissue, the hosts were returned to the incubator until killed 12–17 days after incubation, when they were examined for the presence and state of the donor melanocytes in the manner described above for mice.

DISPOSITION OF MELANOCYTES IN THE PET/MCV MOUSE

Examination of one-day postnatal mice of the PET/MCV strain reveals that melanocytes are found within the connective tissue of many organs. Their distribution is not, however, consistent from litter to litter, or even within the members of the same litter. Melanin-laden pigment cells are seen not only in those tissues previously noted, but also within the lungs, heart, gonads, nucleus pulposus, epiphyses of many of the bones, ribs, and muscles (especially the intercostals and the extremities) (Plate, figs. 1, 2, 3, 5, 7, 8); in the pericardium, serosae, the mesenteries (Plate, figs. 4, 6); in the adventitia of the esophagus, trachea, kidney, blood-vessels, and ducts; and in many other positions. Melanocytes are in fact consistently absent only from the connective tissue of the gut mucosa.

As in the fowl (Reams, 1956), the pigment cells within the connective tissues of the PET/MCV mouse first occur as melanoblasts which had probably migrated from the neural crest. With the onset of melanogenesis they are seen as spherical, unbranched melanocytes which later assume a branched or dendritic shape. That the spherical melanocytes arise from melanoblasts present in the area is indicated by the development of melanocytes in grafts of tissue, taken 2 days prior to their expected appearance, which are grown in the coelom of host chick embryos.

From a study of individuals of different age groups it was found that each area

TABLE 1

Appearance of melanocytes in major regions

Age in days	Areas												
	Semicircular canals	Perineum	Lungs	Intercostales	Kidney	Diaphragm	Pericardium and heart	Gonad-medulla	Ribs and cartilages	Umbilical collar	Skin	Mantle of hind limb	Epiphyses of hind limb
Prenatal	*	*	●	●	*	*	*	*	●	*	*	*	*
14	*	*	●	●	*	*	*	*	●	*	*	*	*
15	*	*	●	●	*	*	*	*	●	*	*	*	*
16	*	*	●	●	*	*	*	*	●	*	*	*	*
17	*	*	●	●	*	*	*	*	●	*	*	*	*
18	*	*	●	●	*	*	*	*	●	*	*	*	*
19	*	*	●	●	*	*	*	*	●	*	*	*	*
20	*	*	●	●	*	*	*	*	●	*	*	*	*
Birth	*	*	●	●	*	*	*	*	●	*	*	*	*
3	*	*	●	●	*	*	*	*	●	*	*	*	*
4	*	*	●	●	*	*	*	*	●	*	*	*	*
8	*	*	●	●	*	*	*	*	●	*	*	*	*
30	*	*	●	●	*	*	*	*	●	*	*	*	*
1 year	*	*	●	●	*	*	*	*	●	*	*	*	*

* Branched melanocytes. ● Unbranched melanocytes.

has its own particular time for the appearance of the melanocytes and for the development of branches by the melanocytes. Table 1 summarizes the time of appearance and the condition of the melanocytes for certain of the areas. To illustrate, from day 15 to day 17 prenatal, the presence of pigment cells in the thoracic wall can be detected only by the dopa technique or by grafting tests. Melanocytes appear scattered in the connective tissue of the intercostal muscles at day 17 of embryonic life. At this time the melanocytes are spherical in form and are laden with melanin granules. Counts of the melanocytes at various ages show that the pigment cells continue to slowly proliferate until day 4 of postnatal life, when they change their shape by developing branches and concomitantly lose their ability to multiply. After this there is little or no increase in the number of melanocytes present among the fibres of the intercostal muscles. The apparent decrease in the number of melanocytes from this stage on into adulthood is due to the increase in size of the region with relatively no change in the number of its melanocytes, as well as to necrosis, since many of the melanocytes are seen in varying stages of degeneration.

Spherical melanocytes are likewise seen in the tissue of the lung on the 17th prenatal day. However, they remain spherical here and maintain their population balance with the quantity of lung tissue even in the adult.

In general, melanocytes are found not only in the skin but also within much of the connective tissues of mice of the PET/MCV strain. Further, the time at which melanin production begins, and/or the production of branches is evoked, is characteristic of the organ in which the pigment cells reside.

TESTS OF THE DEVELOPMENTAL CAPACITY OF SPHERICAL MELANOCYTES

In view of the similarity between the descriptive phases of the PET/MCV mouse investigation and those for the chick (Reams, 1956), the question arises as to whether the melanocytes of this mouse will exhibit a similar developmental capacity and morphogenetic behaviour. Techniques of intracoelomic grafting and grafting to the skin of the leg-bud of host chick embryos offer a means to this end. Since grafts of serosa which contained spherical melanocytes, regardless of the age of the donor (18 days prenatal to 4 days postnatal) or the area (pleural or peritoneal), presented similar results, the data will be grouped for convenience.

A total of twenty-seven intracoelomic grafts of serosa have been recovered and examined. The graft melanocytes and their progeny were generally found scattered within a limited area of the coelomic lining of the abdominal wall and in six cases were buried within the substance of the rump. The great majority of the melanocytes observed in eleven hosts recovered prior to day 15 of incubation were of the spherical type. There had been a fivefold increase in the melanocytes since the time of grafting. Sixteen hosts recovered after the 15th day contained

donor melanocytes which were branched and whose number was essentially no different from those recovered just prior to day 15.

To test the proliferative capacity of the spherical melanocytes of the serosae, intracoelomic grafts were also made of dermis taken from 18-day PET/MCV mouse embryos. The absence of epithelial spheroids and/or hairs in the recovered grafts was used as the criterion for the purity of the original dermal grafts. Although dendritic melanocytes were in most of the grafts, melanoblasts were present also—as evidenced by the great number and wide distribution of melanocytes found in the eleven hosts at recovery (cf. Rawles, 1940, 1947). In respect to the shape of the melanocytes, the data are in agreement with those previously described.

Only 5 out of 24 grafts of serosa with its spherical melanocytes made to the leg-bud could be located upon recovery of the host embryos at 12 days. Since some grafts did take, it might be presumed that many of the grafts had been rejected by the host and lost. However, of those hosts in which mouse pigment cells could be detected, the melanocytes were comparatively few in number and were branched.

Thus it would appear that the spherical melanocytes of the PET/MCV mouse serosae are capable of proliferation but not as rapidly as melanoblasts. In addition, there is a critical time within the host at which the melanocytes branch, regardless of the age of the donor from which the melanocytes were removed.

DISCUSSION

Mice of the PET/MCV strain differ little in external appearance from any other black mouse strain (for example, C_{57} Black). However, internally there is found a wide distribution of melanocytes throughout the connective tissues of the body. If melanocytes may be presumed to be of neural crest origin, then this vividly emphasizes the extent to which the cells of the neural crest may be distributed within the individual. The neural crest has been designated as forming, or contributing to the formation of, a great number of structures (Hörstadius, 1950), particularly in the amphibia. To prove experimentally many of the contributions of the neural crest in amphibia is often difficult and open to doubt. To do so in mammals borders on the realm of the impossible. However, since melanocytes are derived exclusively from the neural crest (for a review of the evidence, see Rawles, 1948) they may be considered here as markers for the distribution of crest cells in the PET/MCV mouse. Considered in this way, the structures within the PET/MCV mouse containing crest cells form an impressive list. The distribution of neural crest cells in other mouse strains might therefore be inferred to be greater than is generally appreciated.

Rawles (1947) demonstrated the progressive spread of neural crest cells in the mouse and showed that the melanocytes of the skin and its derivatives were of neural crest origin. That the pigment cells found in the deeper tissues of PET/

MCV mice are indeed neural crest melanocytes and not melanin-bearing macrophages is shown by several lines of evidence. First, a macrophage must have an extrinsic source of melanin granules to phagocytize before it can become melanin-bearing. In the first sites of the PET/MCV mouse in which pigment-bearing cells appear, the large number of cells to develop granules simultaneously is notable. In addition, just prior to the time at which the black melanin granules appear, dopa-positive cells are to be found in equivalent numbers. If these cells do not manufacture their own melanin granules, whence then are they obtained? Second, the progressive spread of melanoblasts, as demonstrated by grafting experiments and the dopa-method, and later as observed with the melanocytes, is in conformity with the pathways and progress in time one might anticipate. Finally, when grafted into the coelom of host chick embryos, these 'internal' pigment cells show a behaviour identical to that found for coelomic melanocytes of the chick.

In the chick, a melanoblast in its differentiation towards a definitive melanocyte passes through an intermediate phase of either a branched melanoblast or an unbranched melanocyte (Reams, 1956). The sequence of migratory melanoblast to spherical melanoblast to branched melanoblast to branched melanocyte is characteristic of the skin. In other areas the procedure generally is migratory melanoblast to spherical melanocyte to branched melanocyte. The presence of 'dendritic cells' (melanoblasts) in the skin and their subsequent production of melanin have been described by Billingham (1948), Reynolds (1954), and others. A sequence comparable to that described here for the pigment cells within the skin of the chick has been found for the differentiation of melanocytes in human skin (cf. Zimmermann & Becker, 1959). In the present investigation it has been shown that the sequence of morphogenesis for the pigment cells in the PET/MCV mouse is in accord with that found in the chick for the skin and internal tissues.

Since Rawles (1940) demonstrated that the chick coelom was a favourable environment for the growth of mouse tissue, it has been employed for diverse investigations on pigment cell behaviour in the mouse. In all instances host chick embryos containing grafts of mouse tissue were not killed until late in development, and the melanocytes found were of the branched type. The discovery of 'internal' melanocytes in the PET/MCV mouse and the observation that differentiation is similar to that of melanocytes in the chick suggested an investigation of the phases of differentiation of mouse pigment cells within early stages of chick embryo hosts. Hence, grafts of serosa and of dermis were made to chick embryo hosts. These hosts were recovered at varying ages and the condition of the donor pigment cells from the grafts determined. The mouse melanocytes, regardless of whether they were the spherical melanocytes of the serosa or whether they came from the melanoblasts of the dermis, were found to be of the unbranched (spherical) type in all chick hosts prior to day 15 of incubation. After this time, the mouse melanocytes produced branches and became dendritic

in shape. The melanocytes within grafts of mouse skin were found to be branched from the time of their first appearance, without regard to the age of the host. In addition, spherical melanocytes grafted to the skin of chick hosts also branched prior to day 15. Thus it appears that the epidermis has a strong capacity to evoke the branching of pigment cells. In its absence the pigment cells seem to be unable to branch until comparatively late in the development of the host.

That the coelomic lining of the chick holds the production of branches by a pigment cell in check, and that branching is effected only by certain tissues of the embryo, has been shown by Reams (1956). Furthermore, the branching appears to be brought about by a chemical agent which is first effective locally at the site of its production and, later, humorally through a build-up in its concentration in the vascular system (Reams, 1957; Reams, Nichols, & Hager, 1959). That the morphogenesis of the melanocytes of the PET/MCV mouse is brought about in a similar manner is demonstrated by the following evidence.

In the breeds of pigmented fowl examined, melanocytes, when present in the coelomic lining, remain spherical until about day 13 of incubation, at which time they branch. When grafted into the coelom of White Leghorn chick host embryos, these spherical melanocytes produce branches at day 15. It is interesting to note that the time of branching of melanocytes of chick into chick or mouse into chick is the same. In all cases, where grafts were not contaminated with epidermis, the melanocytes branched at a time specific to the host and not in accord with the chronological age of the graft cells. To cite an example, melanocytes in grafts of pleura from an 18-day PET/MCV fetus branched at day 15 in a chick host—after a total of 12 days within the host. The melanocytes are all branched in the pleura of normal PET/MCV mice at day 4 postnatal—an equivalent length of time of about 7 days. Since the method of intracoelomic grafting does not notably slow up the rate of normal growth and differentiation of a graft, it is concluded that there is a real differential of 5 days between the normal and the experimental. Thus the data lead to the assumption that the branching of PET/MCV mouse as well as chick pigment cells is indeed brought about by a chemical entity which is active initially at the site of its production and, later, as its concentration builds up, elsewhere in the body. And, in addition, its action, as observed for chick and mouse, is not species specific.

SUMMARY

1. Mice of the PET/MCV strain are characterized by having melanocytes scattered in the connective tissues of most of the body. The 'internal' pigment cells generally first appear in fetuses as spherical melanocytes which later produce branches and lay down melanin. By contrast, pigment cells within the skin branch prior to the onset of melanogenesis.

2. Spherical melanocytes of the serosae of the PET/MCV mouse proliferate within the coelomic lining of host chick embryos. However, the degree of pro-

liferation and spread is considerably less than that of melanoblasts under similar conditions.

3. Under the conditions of experimental grafting, both spherical 'internal' melanocytes and melanoblasts from the dermis showed a similar pattern of differentiation. The time at which donor melanocytes produce branches within the host chick embryos is in accord with their location and the specific time expected of the host and not of the donor.

4. The data suggest that the branching of a pigment cell is evoked by a morphogenic substance which is produced by certain tissues of the body, particularly by the epidermis. The effect, at least with regard to the mouse and chick, is not species specific, since grafted mouse pigment cells react in keeping with the chick environment.

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EXPLANATION OF PLATE

Fig. 1. Hind-foot of 1-day PET/MCV mouse. Note melanocytes in epiphyses. ×30.

Fig. 2. Marrow portion of centrum of tail vertebra with melanocytes. ×100.

FIG. 3. Melanocytes within muscles of thigh of new-born. ×40.

FIG. 4. Abdominal wall of 18-day PET/MCV fetus showing melanocytes in the peritoneum. ×40.

FIG. 5. Lung of 18-day fetus with unbranched melanocytes. ×100.

FIG. 6. Pigmented pericardium from 3-day PET/MCV mouse. ×100.

FIG. 7. Thoracic wall of 3-day PET/MCV mouse. Melanocytes in intercostales and in costal cartilage. ×100.

FIG. 8. Thoracic wall of 4-day mouse showing branching of melanocytes in intercostals. ×100.

All specimens unstained and cleared in wintergreen oil.

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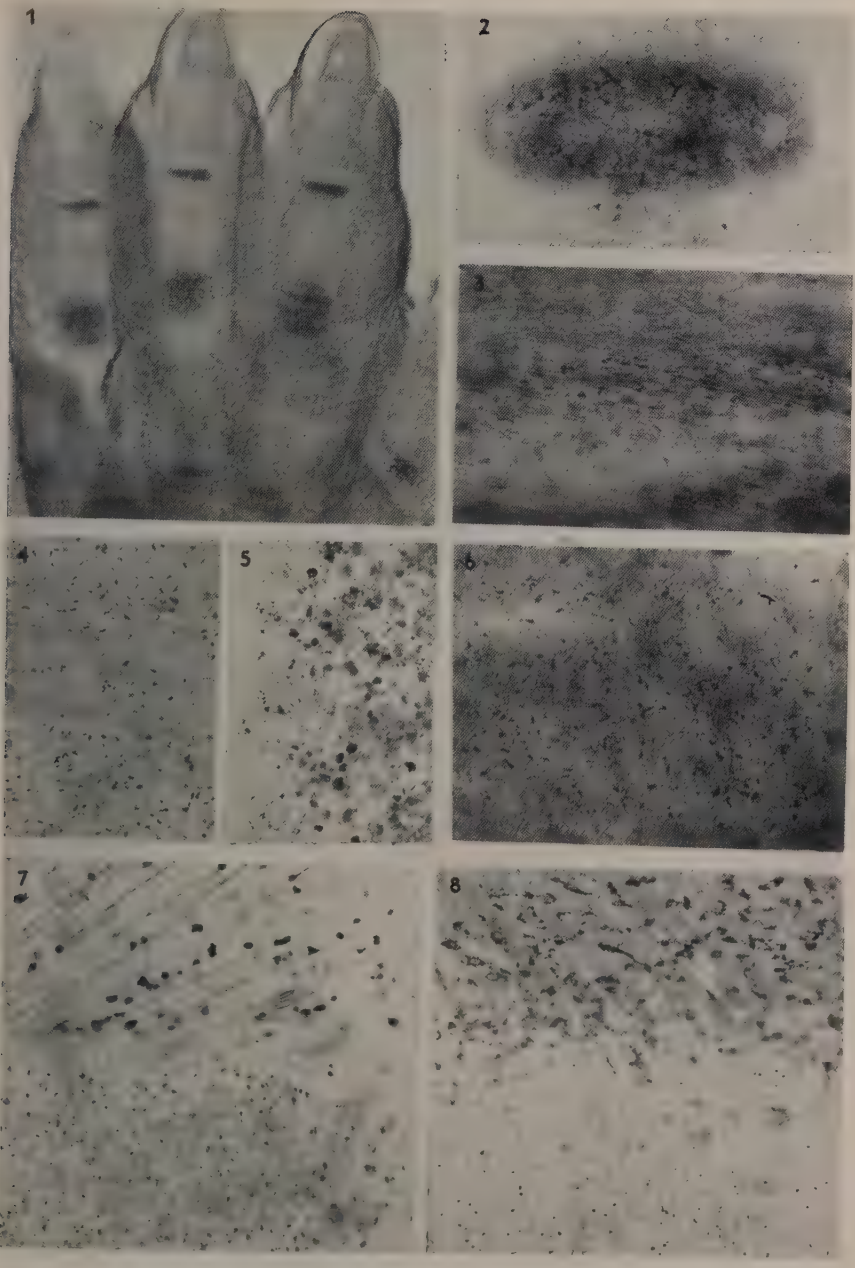

S. E. NICHOLS *and* W. M. REAMS

Induction de cartilage *in vitro* par l'extrait d'otocystes d'embryons de Poulet

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AVEC DEUX PLANCHES

En 1937 De Beer, dans *The Development of the Vertebrate Skull*, posait le problème suivant: 'Can extracts of auditory vesicles induce the formation of cartilage *in vitro*? *in vivo*?' (p. 514). Nous pensons avoir trouvé une réponse à la première question: l'extrait d'otocystes induit *in vitro* la formation de cartilage à partir du mésenchyme otique, chez l'embryon de Poulet.

Des recherches antérieures nous avaient montré que l'épithélium auditif est l'inducteur normal de la capsule otique cartilagineuse (Benoit, 1955). D'autre part, lorsque l'ébauche otique épithéliale est déplacée au niveau des futures régions méningées et dermiques, le mésenchyme reste indifférent (Benoit, 1956). Certains territoires du mésenchyme céphalique sont donc incapables de répondre à l'induction otique. De même le mésenchyme somitique associé à un otocyste *in vivo* ou *in vitro*, ne se différencie jamais en cartilage (Benoit, 1960). Nous n'avons donc pu éprouver que le mésenchyme otique, en culture *in vitro*, sur un milieu renfermant l'extrait d'otocystes.

Nous avons choisi du mésenchyme et des otocystes de 5 jours d'incubation pour diverses raisons. Le mésenchyme commence à se différencier en pré-cartilage, *in vivo*, dès l'âge de 6 jours. D'autre part, le mésenchyme de 4 jours se cultive mal et se disperse rapidement sur le milieu. Le stade intermédiaire de 5 jours, en revanche, fournit des explants qui se cultivent bien. Cependant l'absence de différenciation de ce mésenchyme n'exclut pas un certain degré de détermination par l'inducteur otique, comme nos expériences de contrôle le montreront. D'autre part, l'ébauche épithéliale de 5 jours nous a semblé susceptible de fournir le maximum de substance active.

TECHNIQUE

Nous avons utilisé la méthode de culture *in vitro* de Wolff & Haffen (1951,

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1952). Le milieu W mis au point par Et. Wolff & Em. Wolff (1952) a été légèrement enrichi.

L'extrait d'otocystes a été préparé à partir d'un microbroyat de 150 à 200 otocystes de 5 jours avec une quantité égale de liquide de Tyrode. Ce broyat a été centrifugé pendant 15 minutes avec une accélération de 4.800 g. Le surnageant a été incorporé au milieu de culture, de telle sorte que le milieu expérimental et le milieu témoin aient la même valeur nutritive et la même consistance.

Le mésenchyme otique a été prélevé à 5 jours d'incubation, dorsalement par rapport à la veine cardinale antérieure, pour éviter d'inclure du mésectoderme viscéral. En avant de l'oreille, le territoire à exciser s'arrête au ganglion de Gasser. En arrière, il est limité par le premier somite. Chaque explant est constitué par un ou plusieurs blocs de mésenchyme pré- ou post-otique. Il est possible de cultiver jusqu'à 5 ou 6 explants par salière pendant une douzaine de jours. Les coupes à 5 ou 7 μ ont été colorées à l'hémalun-éosine.

RÉSULTATS EXPÉRIMENTAUX

Le tableau 1 résume nos observations.

TABLEAU 1

Structure histologique	*Nombre d'explants expérimentaux*	*Nombre de témoins*
Cartilage	5	0
Précartilage (2)	14 (précartilage normal)	9 (précartilage anormal)
Précartilage (1)	7	4
Mésenchyme	4	17
	30	30

Au moment du prélèvement, à 5 jours, le mésenchyme est au stade indifférent, comme le montre la fig. G, planche 2.

1. *Explants cultivés sur extrait d'otocystes*

Ces explants offrent une gamme de différenciations allant du cartilage typique au jeune précartilage (1).

Le cartilage représenté fig. A, planche 1, apparaît sous forme d'un bloc, où la substance fondamentale est nettement visible. Latéralement le mésenchyme est resté indifférent. Notons qu'il n'a plus l'aspect rencontré au début de l'explantation. Il est dense et irrégulièrement parsemé de noyaux.

Le précartilage (2) est fréquent dans cette série (fig. B, planche 1). On note la présence d'amas bien délimités, où les cellules sont plus ou moins serrées et encore anastomosées. Il manque la substance fondamentale.

Le précartilage (1) (fig. C, planche 1) est constitué par une zone de cellules

serrées à noyaux arrondis. D'un côté, la limite est bien marquée entre ce jeune précartilage et le mésenchyme indifférent qui le borde.

A côté de ces explants, qui ont poursuivi plus ou moins loin leur évolution, un petit nombre est resté à l'état mésenchymateux.

2. *Témoins*

Les témoins n'ont jamais évolué en cartilage typique.

Le précartilage (2) (fig. D, planche 2) est pauvre en cellules. Sa texture lâche, presque lacunaire, semble indiquer une certaine dégénérescence.

Le précartilage (1) ressemble à celui qui a été observé chez les explants du premier groupe. Sur la fig. E, planche 2, il apparaît du côté droit, sous forme d'une plage allongée et recourbée. Le mésenchyme indifférent est abondant.

Enfin de nombreux explants (17 sur 30) sont restés totalement à l'état mésenchymateux (fig. F, planche 2).

Ces résultats démontrent que le mésenchyme de 5 jours est déjà suffisamment déterminé pour amorcer sa différenciation. Mais le stade du précartilage (2) marque une limite qui n'est jamais dépassée, et semble difficilement atteinte. La comparaison des deux séries de cultures permet de faire plusieurs remarques (voir le tableau).

Le cartilage typique apparaît seulement dans la série expérimentale.

Les explants évoluent dans chaque série suivant des gradations inverses. Dans la série expérimentale, le mésenchyme est peu fréquent (4 cas sur 30) et les structures différenciées le sont nettement plus (26 cas). Dans la série de contrôle, c'est le mésenchyme qui apparaît le plus souvent (17 cas sur 30). Les explants différenciés sont relativement moins nombreux (13 cas), et les structures les plus évoluées sont anormales (précartilage (2)).

Ces constatations permettent de conclure que la présence d'extrait d'otocystes dans le milieu de culture, fait nettement progresser la différenciation des explants expérimentaux, par rapport aux témoins. L'activité de cet extrait est relativement modérée, si on la compare à celle de l'épithélium auditif cultivé en présence de mésenchyme. Il nous est, en effet, arrivé de prélever accidentellement un peu d'épithélium otique en même temps que le mésenchyme. Dans tous les cas, il se formait du cartilage typique autour du rudiment membraneux.

On peut donc conclure que l'épithélium auditif élabore une substance diffusible, qui induit le mésenchyme céphalique à se différencier en cartilage, ou qui renforce la détermination de ce mésenchyme à évoluer en cartilage, lorsqu'il a subi un début d'induction.

DISCUSSION

Le mode d'action de l'organisateur chez les embryons d'Amphibiens a reçu plusieurs explications. L'hypothèse chimique a la faveur de la majorité des chercheurs. Elle implique l'existence d'une ou de plusieurs organisines diffu-

sibles, émises par le chordo-mésoderme et déterminant la différenciation du neurectoderme. En 1953, Niu & Twitty ont démontré l'existence d'une substance diffusible en culture *in vitro*. Ces auteurs avaient fait séjourner l'organisateur pendant une semaine dans le milieu de culture, où l'ectoderme de gastrula a été ensuite transporté. Nous avons employé une autre méthode. La réserve de substance diffusible a été préparée par centrifugation d'un broyat d'otocystes. L'extrait a été incorporé au milieu solide de Wolff & Haffen (1951, 1952). Malgré la petite quantité d'extrait obtenu et sa faible concentration, nous sommes arrivé à démontrer l'activité d'une organisine, qui induit le mésenchyme otique à évoluer en cartilage (Benoit, 1959).

Divers auteurs, qui ont étudié des sujets voisins, contestent l'existence d'une substance diffusible dans l'induction du cartilage. Citons Grobstein & Parker (1954), Grobstein & Holtzer (1955), Lash, Holtzer & Holtzer (1957), qui pensent à un facteur non soluble dans l'eau transmis par la substance interstitielle des tissus. Ces conclusions sont tirées principalement d'expériences, où des filtres de porosité et d'épaisseur variables sont intercalés entre un fragment de moelle et des somites cultivés *in vitro*. Grobstein (1956, 1957) et Grobstein & Dalton (1957) étaient d'ailleurs arrivés à des résultats analogues en étudiant l'induction des tubes du métanéphros par la moelle, au moyen de la méthode des filtres. En conclusion, l'existence d'un facteur transmis de l'inducteur au tissu réacteur est reconnu. Mais pour les auteurs cités, ce facteur n'est pas soluble, bien qu'il ne semble pas contradictoire de l'admettre. Strudel (1959) vient d'ailleurs d'obtenir un résultat analogue au nôtre, dans le même laboratoire. Il a extrait, à partir d'un microbroyat de moelle et de chorde, une substance diffusible qui induit, *in vitro*, les somites à se différencier en cartilage.

D'une manière générale, on peut se demander si le cartilage est induit par une ou plusieurs substances solubles.

Des problèmes analogues se posent à propos des travaux de plus en plus nombreux depuis Nageotte (1918), concernant certains tissus, vivants ou dévitalisés, ou leurs extraits, qui sont capables d'induire la formation de cartilage à partir de cellules conjonctives *in vivo* ou de fibroblastes *in vitro*. Citons quelques auteurs: Fischer (1931), Törő (1935), Levander (1938, 1945, 1949), Nassanov (1934) et Bridges & Pritchard (1958). Il ne semble pas y avoir de relation entre les inducteurs embryonnaires et les inducteurs hétérogènes de l'adulte.

RÉSUMÉ

Chez l'embryon de Poulet, un extrait d'otocystes est capable d'induire la formation de cartilage à partir du mésenchyme otique, cultivé *in vitro*, ou de renforcer la détermination de ce mésenchyme. On peut donc admettre que l'induction du cartilage otique s'effectue au moyen d'une substance diffusible.

SUMMARY

In the chick embryo an extract of otocysts can induce cartilage formation in otic mesenchyme, cultured in vitro, or can reinforce the determination of such mesenchyme. It may therefore be suggested that the induction of otic cartilage is produced by a diffusible substance.

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EXPLICATION DES PLANCHES

Planche 1

Fig. A. Explant expérimental différencié en cartilage. ×200.
Fig. B. Explant expérimental différencié en précartilage (2). ×140.
Fig. C. Explant expérimental différencié en précartilage (1). ×360.

Planche 2

Fig. D. Explant témoin différencié en précartilage (2) anormal. ×250.
Fig. E. Explant témoin différencié en précartilage (1). ×210.
Fig. F. Explant témoin resté à l'état de mésenchyme. ×130.
Fig. G. Mésenchyme otique au moment du prélèvement. ×420.

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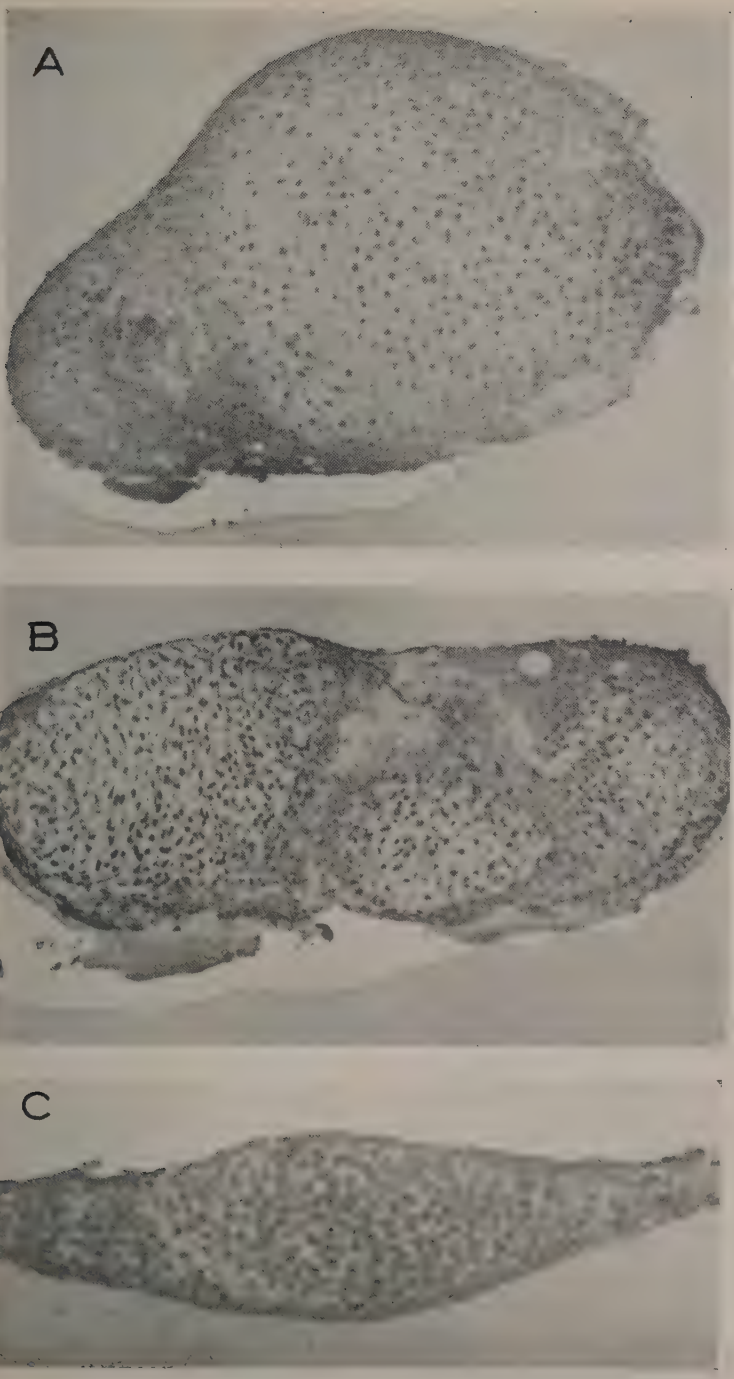

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Planche 1

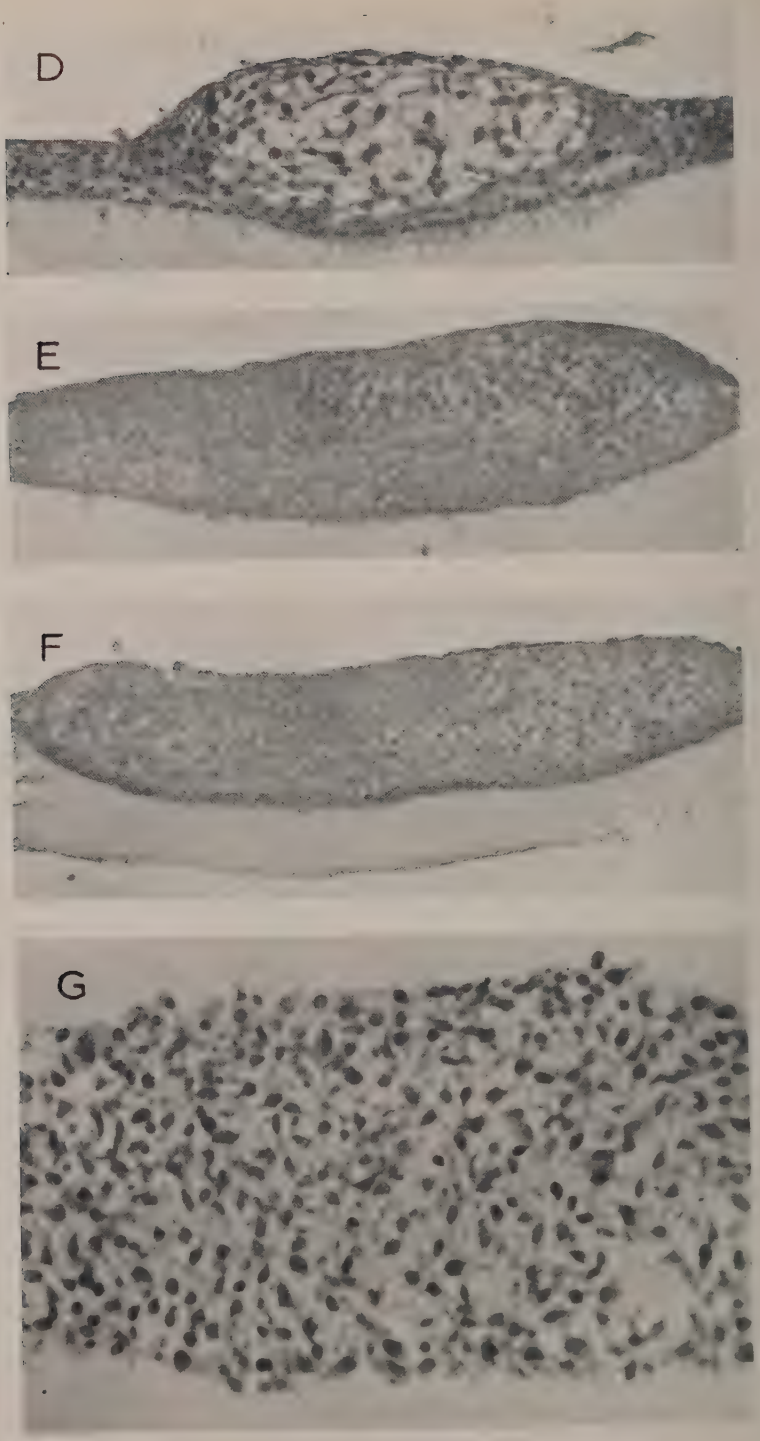

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Planche 2

L'Otocyste exerce-t-il une action inductrice sur le mésenchyme somitique chez l'embryon de Poulet?

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AVEC DEUX PLANCHES

L'ÉPITHÉLIUM auditif induit normalement le cartilage de la capsule otique, à partir du mésenchyme céphalique, chez l'embryon de poulet (Benoit, 1955). Ce pouvoir inducteur peut-il s'exercer à l'égard du mésenchyme axial du tronc? Nous avons tenté de résoudre ce problème en greffant des otocystes au contact des somites, ou en associant otocystes et somites en culture *in vitro*.

EXPÉRIENCES DE GREFFES

La méthode consiste à implanter un otocyste, soigneusement débarrassé de toute trace de mésenchyme, à la place de la moelle et la chorde d'un hôte âgé de 48 heures (15 à 20 somites). Strudel (1953 *a*, *b*) avait montré que la suppression de la moelle et la chorde sur une longueur de 10 somites, jusqu'au stade de 30 somites, entraînait l'absence totale de cartilage vertébral. Watterson, Fowler & Fowler (1954) avaient obtenu le même résultat jusqu'au stade de 28 somites. Par conséquent chez nos embryons, plus jeunes que ceux des auteurs cités, le réacteur somitique est au stade indéterminé.

L'ébauche otique prélevée sur un donneur âgé de 2 ou 3 jours, est débarrassée du mésenchyme qui l'entoure par des moyens microchirurgicaux, ou par l'action de la trypsine diluée, selon une technique inspirée de celle de Moscona (1952). Il est absolument essentiel que le greffon soit purement épithélial, sous peine d'adjoindre un peu de réacteur normal céphalique, au réacteur hétérogène somitique qu'il s'agit d'éprouver. En revanche cette association sera utilisée comme contre-expérience.

Tous les embryons élevés jusqu'à 9 jours, ont été colorés en masse au bleu de méthylène pour mettre en évidence les éléments cartilagineux, d'après la technique de Lundvall. Les coupes ont été colorées à l'hémalun-éosine.

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Résultats expérimentaux

Première série

Greffon: cupule otique ou jeune vésicule (2 à 3 jours d'incubation) débarrassée au microscalpel du mésenchyme qui l'entoure.

Hôte: au stade de 15 à 20 somites, privé de moelle et de chorde sur une longueur de 10 somites environ.

Dix embryons ont été sacrifiés à 9 jours d'incubation. La coloration au bleu de méthylène ne révèle aucune trace de cartilage dans la région, variable suivant les sujets, où la colonne vertébrale est interrompue. L'étude histo-

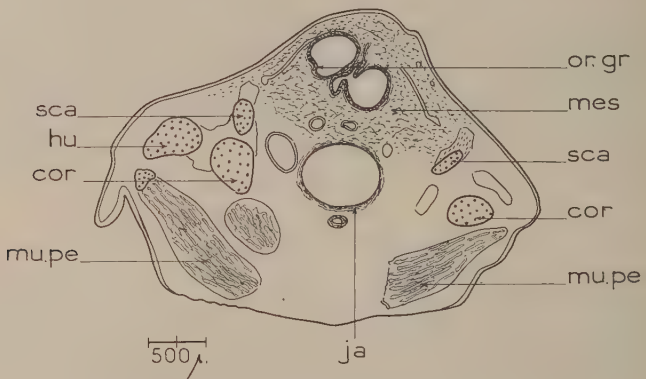

FIG. 1. *cor*, coracoïde; *hu*, humérus; *ja*, jabot; *mes*, mésenchyme; *mu. pe*, muscle pectoral; *or. gr*, oreille greffée; *sca*, scapulum.

logique confirme cette observation. L'exemple représenté par la fig. 1 et la fig. A, planche 1 montre certains éléments des ceintures pectorales et la base d'une aile. Au-dessus du jabot, la moelle, la chorde et les vertèbres font complètement défaut. A leur place se trouve le greffon otique, anatomiquement bien différencié en labyrinthe membraneux. Il est entouré de mésenchyme lâche, sans aucune trace de différenciation en cartilage.

Deuxième série

Greffon: vésicule otique débarrassée du mésenchyme céphalique après passage dans la trypsine à 1/500 pendant 4 minutes.

Hôte: préparé comme dans la série précédente.

Les 9 embryons étudiés ne montrent aucune trace de cartilage surnuméraire après coloration au bleu de méthylène. A l'examen microscopique, l'absence de cartilage autour de l'oreille est confirmée dans tous les cas. Dans l'exemple choisi (fig. 2 et fig. B, planche 1) le greffon apparaît à l'emplacement des organes axiaux, sous forme d'une vésicule portant un faible diverticule.

L'oreille membraneuse est entourée de mésenchyme lâche. Les résultats précédents se trouvent confirmés.

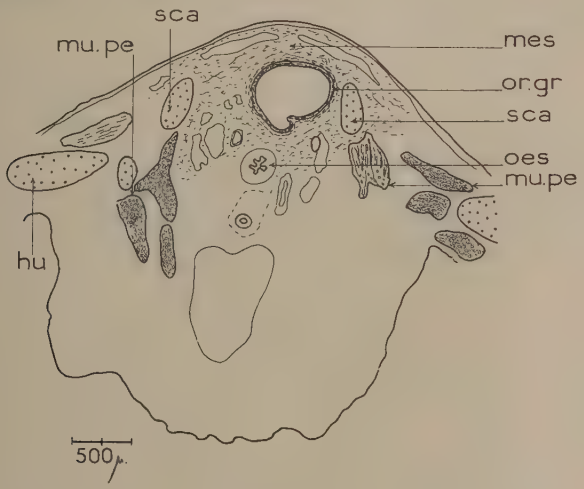

FIG. 2. *hu*, humérus; *mes*, mésenchyme; *mu. pe*, muscle pectoral; *oes*, œsophage; *or. gr*, oreille greffée; *sca*, scapulum.

Troisième série (contre-expérience)

Greffon: cupule otique; un peu de mésenchyme céphalique adhérent a été conservé.

Hôte: semblable aux précédents.

Cette 3ème série d'expériences a pour but de rechercher le pouvoir inducteur du greffon en position hétérotopique, lorsqu'il est associé à un fragment de réacteur mésenchymateux céphalique normal.

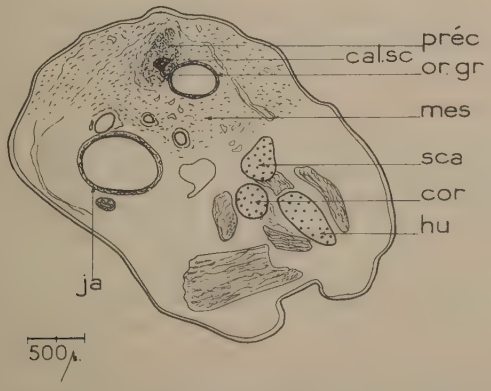

FIG. 3. *cal. sc*, canal semi-circulaire; *cor*, coracoïde; *hu*, humérus; *ja*, jabot; *mes*, mésenchyme; *or. gr*, oreille greffée; *préc*, précartilage; *sca*, scapulum.

Chez 11 opérés, âgés de 9 jours, colorés au bleu de méthylène, on aperçoit une ou plusieurs taches d'un bleu très pâle dans la région où les vertèbres sont absentes. L'examen histologique révèle dans tous les cas que ces taches correspondent à du cartilage. La fig. 3 et la fig. C, planche 1 représentent l'oreille greffée à l'emplacement de l'axe vertébral au-dessus du jabot. Elle est en forme de vésicule entourée de mésenchyme. Un canal semi-circulaire bien différencié est nettement encapsulé. Notons que le tissu induit est encore à l'état de précartilage, marquant un certain retard par rapport au développement normal.

Cette série expérimentale démontre que l'oreille greffée en position hétérotopique conserve son pouvoir inducteur. D'autre part il a suffi de conserver un fragment de mésenchyme céphalique pour que du cartilage apparaisse.

Conclusion

La corrélation est bien établie entre l'absence (séries 1 et 2) ou la présence (série 3) de mésenchyme otique et l'absence ou la présence de cartilage. Donc le mésenchyme axial du tronc est incompétent pour répondre à l'action inductrice de l'oreille interne.

Ichikawa (1935) obtint des résultats comparables chez le triton. Un débat avait été ouvert entre Sternberg (1924), Balinsky (1925, 1927), Filatow (1927) et Yntema (1933) d'une part, et Stone (1926), Kaan (1926, 1930) et Luther (1927) d'autre part. Les premiers soutenaient que le mésoderme non céphalique pouvait évoluer en cartilage sous l'action inductrice de l'oreille. Les seconds défendaient le point de vue contraire. Ichikawa démontra que le cartilage apparaissait dans n'importe quelle situation hétérotopique, pourvu que l'oreille fût accompagnée de mésenchyme céphalique, qui était seul compétent.

CULTURES *IN VITRO*

La méthode des cultures *in vitro* de Wolff & Haffen (1951, 1952) nous a permis d'associer l'otocyste à des somites et de retrouver les résultats précédents. En outre, un aspect intéressant de la chondrogénèse a été mis en évidence.

D'une manière générale les otocystes explantés sont âgés de 3 jours et débarrassés du mésenchyme après passage dans la trypsine diluée. Chaque otocyste est entouré d'une rangée de somites prélevés dans la région moyenne de donneurs au stade de 15 à 23 somites. Les cultures ont duré en moyenne une dizaine de jours.

L'épreuve préalable du milieu de culture, en vue d'étudier la morphogénèse normale des ébauches, présente une grande importance. Il est indispensable d'apprécier le pouvoir de l'inducteur otique et la capacité de réponse du réacteur somitique, cultivés séparément sur un milieu donné. A cet effet l'otocyste est explanté avec le mésenchyme qui l'entoure, et les somites le sont avec la moelle et la chorde.

Nous avons étudié la valeur du milieu W, mis au point par Em. Wolff (1952). Lorsqu'on explante des otocystes de 3 jours entourés de mésenchyme, il se forme du précartilage dans quatre cas sur onze. Dans le cas d'explantation de tronçons axiaux de 2 jours (19 à 25 somites) comprenant moelle, chorde et somites, la chondrogénèse est un peu meilleure. Il se forme 4 fois sur 10, soit du cartilage typique, soit plus rarement du précartilage.

Il est possible de doubler la valeur nutritive du milieu, tout en lui conservant à peu près la même consistance. Lorsqu'on cultive des otocystes, avec le mésenchyme adhérent, sur ce milieu enrichi, il se développe du précartilage dans tous les cas. De même la culture de tronçons axiaux de donneurs ayant 18 à 24 somites, permet d'obtenir dans tous les cas du cartilage ou moins fréquemment du précartilage.

Il semble donc que l'action de l'inducteur sur le réacteur, qui détermine l'apparition de cartilage, soit une condition nécessaire mais non suffisante en culture *in vitro*. Encore faut-il que la valeur nutritive du milieu soit suffisante. Précisons qu'il s'agit de jeunes ébauches. Pour le mélange naturel complexe que nous utilisons une simple variation quantitative est efficace. Wolff, Haffen & Wolff (1953) et Stenger-Haffen (1957) avaient fait des observations du même ordre. Les anomalies de différenciation des organes sexués embryonnaires, cultivés *in vitro*, sont en rapport avec la nature du milieu nutritif. Em. Wolff (1957) montre également le rôle de la nutrition, dans la différenciation en cartilage de la syrinx d'oiseau.

Résultats expérimentaux

Ces données de base étant acquises, nous avons réalisé, sur le milieu le plus favorable, trois séries d'explantations concernant l'expérience, la contre-expérience et le contrôle.

Première série

Otocyste de 3 jours associé à des somites prélevés chez des donneurs au stade de 14 à 23 somites.

Douze explants ont été étudiés. La différenciation de l'oreille membraneuse est bonne en général. Le mésenchyme a l'aspect d'un tissu plus ou moins compact: le cartilage est toujours absent. On note souvent l'existence de tégument bien kératinisé. Quelquefois des tubules rénaux apparaissent. Ces détails montrent que les somites étaient en général accompagnés de leur revêtement ectoblastique et qu'un peu de matériel latéral a pu être prélevé. L'explant représenté fig. D, planche 2 comprend côte à côte l'épithélium auditif et du mésenchyme non différencié.

Deuxième série

Otocyste de 3 jours avec un fragment de mésenchyme céphalique, en association avec des somites (stades 14 à 23 somites).

Dans 9 cas sur 11 le labyrinthe auditif est partiellement entouré de précartilage ou de cartilage. On trouve également du mésenchyme indifférent de la peau kératinisée et des tubules rénaux comme dans la série précédente. La fig. E, planche 2 montre une coupe passant au niveau du labyrinthe auditif membraneux et du précartilage qui l'entoure en partie. Aux deux pôles de l'explant apparaissent du mésenchyme, à cet endroit peu abondant, et un tubule rénal.

Troisième série

Somites isolés (stade de 14 à 23 somites).

Le mésenchyme occupe la presque totalité des 10 explants étudiés. Il est dense, en général, et ne présente aucune trace de différenciation en précartilage. Nous retrouvons de la peau kératinisée et de rares tubules rénaux. La fig. F, planche 2 illustre un de ces résultats où l'épiderme en forme de crosse est en voie de kératinisation.

Conclusion

Les cultures *in vitro* aboutissent aux mêmes résultats que les greffes hétérotopiques. Il y a corrélation entre l'absence (série 1) et la présence (série 2) de mésenchyme céphalique et l'absence ou la présence de cartilage. Le mésenchyme somitique reste indifférent. Le milieu de culture n'est pas en cause, puisque les somites se différencient normalement en cartilage en présence de la moelle et la chorde. Nos recherches préliminaires n'avaient pas d'autre but que d'assurer les meilleures conditions, dans lesquelles se fait la chondrogénèse. D'autre part l'examen des témoins (série 3) montre que les somites ont été prélevés à l'état indéterminé. Le stade de 23 somites, auquel nous nous sommes arrêté, n'est pas trop âgé. Cette constatation est conforme aux résultats des expériences *in vivo* en position orthotopique de Strudel (1953 *a*, *b*, 1955) et Watterson, Fowler & Fowler (1954) qui fixent à 30 ou 28 somites le stade où les somites sont encore indéterminés, ou incomplètement déterminés à évoluer en cartilage.

Cependant de nombreux travaux ont montré que des somites plus jeunes, placés hors de leur position normale, pouvaient s'autodifférencier en cartilage. En greffes chorio-allantoïdiennes ou cœlomiques, Hoadley (1925), Murray & Selby (1933), Avery, Chow & Holtzer (1956) et Seno & Büyüközer (1958) ont observé du cartilage à partir de somites isolés provenant de donneurs qui avaient au plus 20 somites. En culture *in vitro* Avery, Chow & Holtzer (1956) et Lash, Holtzer & Holtzer (1957) ont constaté l'apparition de cartilage par autodifférenciation de somites prélevés dès les stades de 18 ou 20 somites. Dans tous ces exemples il est vraisemblable que l'évolution des somites a été influencée par les conditions du milieu anormal qui a été choisi, ce qui pose d'ailleurs un, ou des problèmes. Nous nous en tiendrons donc aux références déjà citées de Strudel (1953 *a*, *b*, 1955) et Watterson, Fowler, & Fowler (1954).

Les somites que nous avons explantés entre les stades de 14 à 23 somites étaient bien au stade indéterminé et ne peuvent en aucune façon être à l'origine du cartilage observé dans notre 2ème série d'expériences. Le mésenchyme somitique est incompétent, *in vitro*, comme il l'a été *in vivo*, à l'égard de l'inducteur otique.

RÉSUMÉ

Lorsqu'une ébauche otique purement épithéliale, cupule ou vésicule, est mise au contact, *in vivo* ou *in vitro*, de somites au stade indéterminé, il ne se produit jamais de cartilage. Inversement le cartilage se forme toujours à partir d'un fragment de mésenchyme céphalique accompagnant l'otocyste. Donc l'épithélium auditif conserve son pouvoir inducteur hors de son emplacement normal, mais le réacteur somitique est incompétent. En culture *in vitro*, la chondrogénèse résultant d'un effet inducteur dépend également de la valeur nutritive du milieu.

SUMMARY

When a purely epithelial otic rudiment of a chick embryo, cup-shaped or vesicular, is put *in vivo* or *in vitro* into contact with somites which are still in an undetermined state, no production of cartilage results. Conversely, cartilage always forms from a fragment of head mesenchyme accompanying the otocyst. Hence the otic epithelium maintains its inductive power when it is outside its normal position, but the somite is not competent to react to it. *In vitro*, chondrogenesis resulting from an inductive effect also depends on the nutritive value of the medium.

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EXPLICATION DES PLANCHES

PLANCHE 1

FIG. A. Microphotographie correspondant à la fig. 1.

FIG. B. Microphotographie correspondant à la fig. 2.

FIG. C. Microphotographie correspondant à la fig. 3.

PLANCHE 2

FIG. D. Association otocyste-somites en culture *in vitro*. ×175. *ea*, épithélium auditif; *m*, mésenchyme (le mésenchyme somitique m est resté indifférent).

FIG. E. Association otocyste-mésenchyme céphalique-somites. ×95. *ea*, épithélium auditif; *m*, mésenchyme; *pc*, précartilage; *r*, tubule rénal. (Le mésenchyme céphalique a évolué en précartilage pc, le mésenchyme somitique m est resté indifférent.)

FIG. F. Somites (+ mésoderme latéral et ectoderme). ×160. *ep*, épiderme en voie de kératinisation; *m*, mésenchyme, *r*, tubule rénal. (Le mésenchyme somitique m, chez ce témoin, est resté indifférent.)

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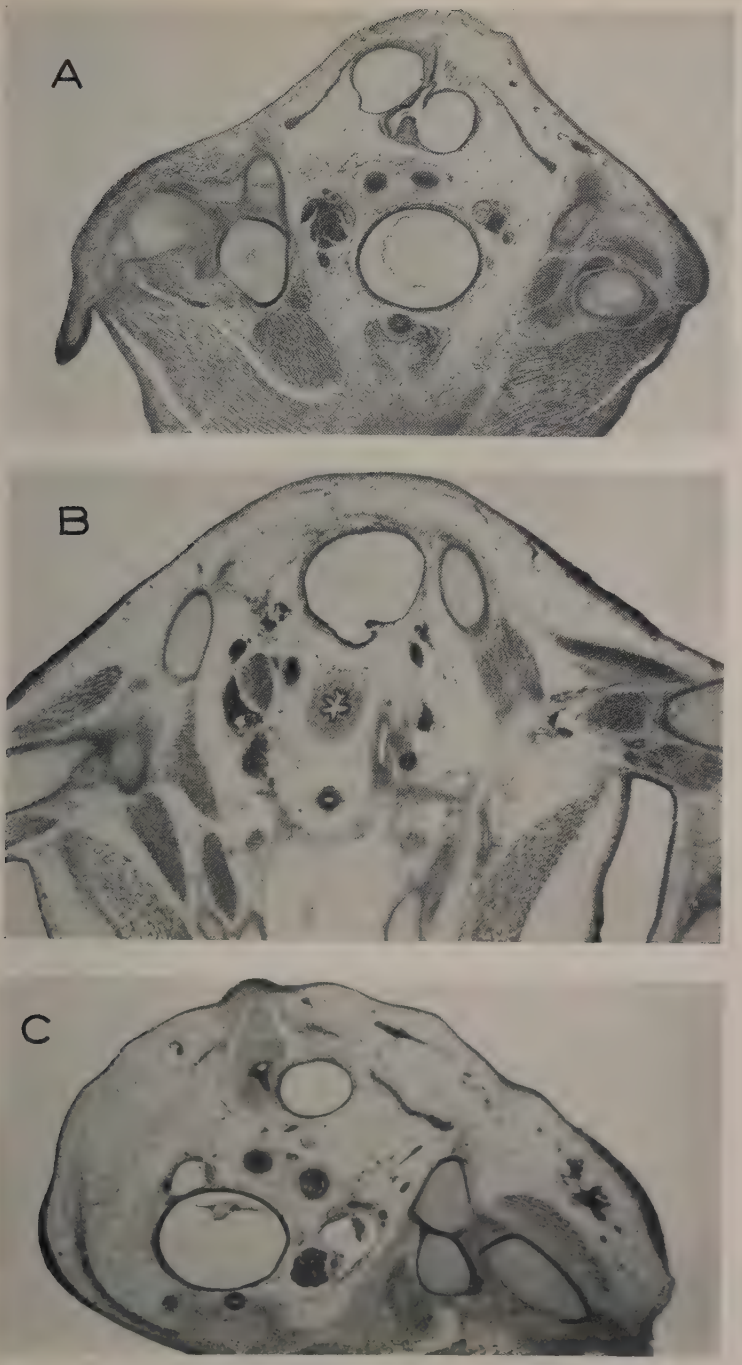
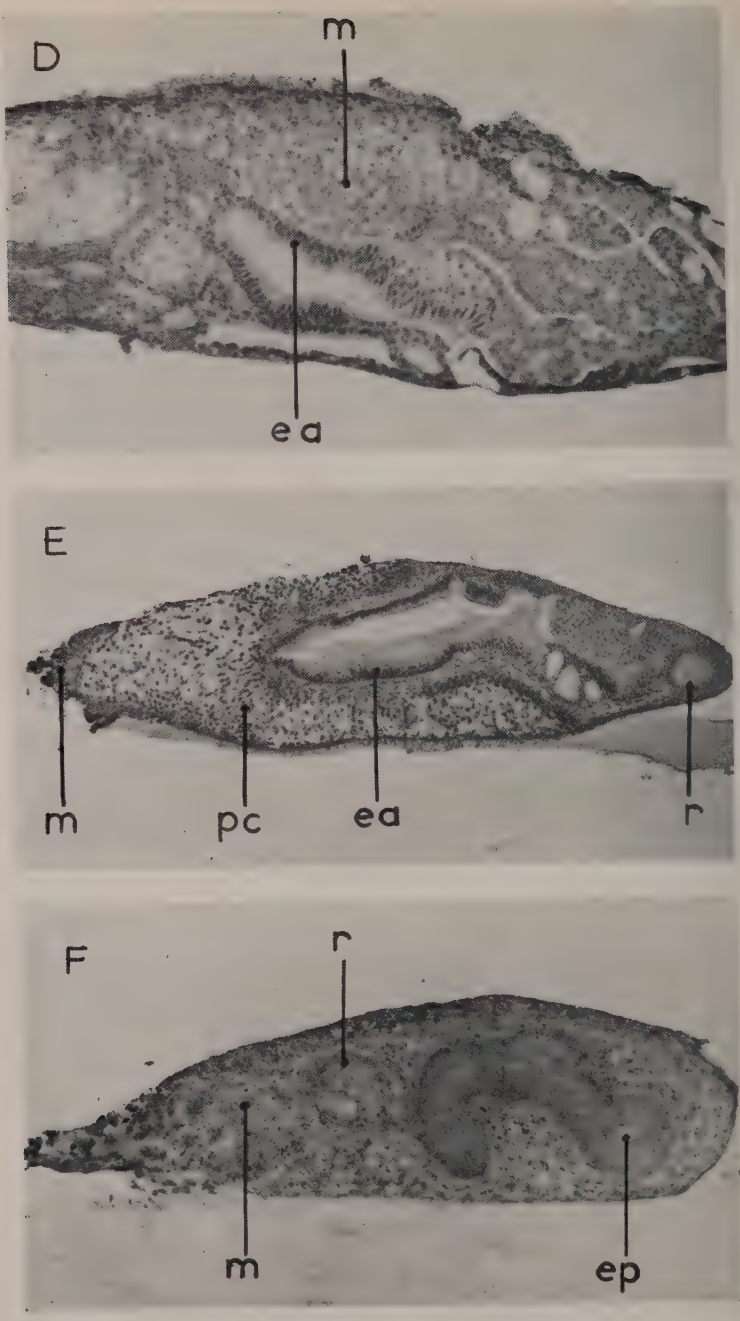


J. A. A. BENOIT

Planche 1



J. A. A. BENOIT

Planche 2

Changes in the Protein Content of Yolk during Chick Embryogenesis

by W. M. McINDOE[1]

From the Agricultural Research Council Poultry Research Centre, Edinburgh

THE original purpose of the present investigation was to determine the rate of degradation of phosphoprotein in the yolk at different times during embryogenesis as a preliminary to examining the mechanism of its assimilation, and to see if phosphoprotein and other proteins were assimilated at different times or at markedly different rates. The early work on the assimilation of yolk protein has been reviewed by Needham (1931) and few pertinent papers have appeared since then (Schenck, 1932; Rupe & Farmer, 1955; Walter & Mahler, 1958). Recently Holoubek & Brada (1956) have made an extensive examination of the distribution of phosphorus compounds in the egg during embryogenesis.

The results of some of the earlier work are difficult to interpret for the following reasons: (*a*) it is not possible to decide if the yolk sac has been included in the analyses of 'yolk'; (*b*) the methods of expressing the results make it impossible to calculate changes in absolute amount; (*c*) apparently eggs from the same hen or group of hens are not used for estimates at different times of incubation. This tends to obscure changes, since fertile eggs from individual hens have different compositions and patterns of embryogenesis (see, for example, Romanoff & Romanoff, 1949; Bernier, Taylor, & Gunns, 1951; Erasmus, 1954). In the present work a group of eight hens has been used throughout and the yolk analysed after separation from the yolk sac.

MATERIALS AND METHODS

Fertile eggs were obtained from an inbred line of Brown Leghorns in their first laying year, the male being from a second inbred line of the same breed.

Eggs which had been incubated for 12–18 days were carefully opened and the contents gently transferred to a Petri dish. The allantoic and amniotic fluids and the albumen were removed and the embryo and membranes separated from the yolk sac, which was rinsed with a little 0·9 per cent. saline. The yolk sac was opened and spread out on the Petri dish. The thick yolk was washed free with the aid of a stream of warm saline (from a plastic wash-bottle) and gentle teasing of the yolk sac, particular care being taken to remove all yolk

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from the folds. The yolk preparation was filtered through a small pad of cotton wool to remove any fragments of yolk sac which might be present. Eggs incubated for 9 days were handled slightly differently, the yolk being more fluid and the yolk sac more fragile. These eggs were opened so that the amniotic and allantoic fluid and much of the white (now quite turgid and immiscible with yolk) could be removed before piercing the yolk sac and collecting the fluid contents. The yolk sac was then separated and treated as before to remove the thick yolk, which was added to the fluid fraction. Hatched chicks (after 21 days' incubation) were killed by cervical dislocation and the yolk sac removed and treated in the same way as that from 12- to 18-day embryos. The wet weight of yolk was determined from weight of yolk sac with and without its contents.

Chemical determinations

One-tenth of the yolk was used for analysis. Trichloroacetic acid (50 per cent.) was added to the yolk suspension to give a final concentration of 5 per cent. After 15 minutes at 0° C. the protein was centrifuged down and washed with 20 ml. 5 per cent. trichloroacetic acid. The acid extract was analysed for total nitrogen, and total and inorganic phosphorus. To remove lipids the protein was mixed with 20 ml. ethanol and allowed to stand overnight, centrifuged, and washed three times with 20 ml. ethanol : ether (3 : 1 v./v.) and finally with ether. The dried protein was dissolved in 0·25 N NaOH and its nitrogen and phosphorus estimated. Contamination of the protein with nucleic acid may be expected to be small since the nucleic acid content of yolk is very low (Solomon, 1957).

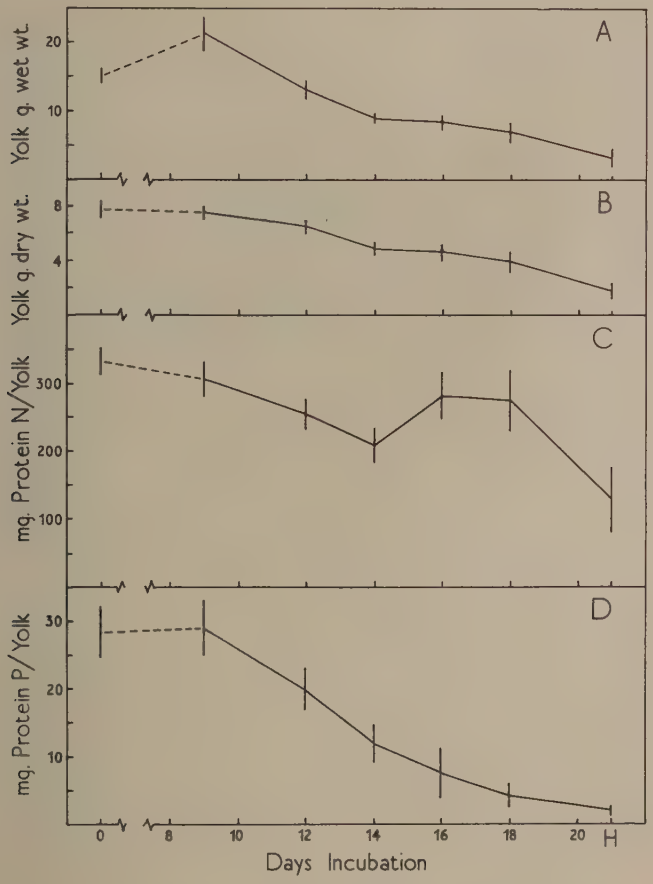
Nitrogen was estimated by the micro-Kjeldahl procedure and phosphorus by the method of Allen (1940). The dry weight of yolk was determined by transferring one-tenth of the yolk suspension to a small Petri dish and removing most of the moisture in an incubator at 37° C. and the remainder in a vacuum desiccator over phosphorus pentoxide.

RESULTS

The results on the composition of yolk at different periods of embryogenesis are given in Text-fig. 1. The pattern of changes in wet and dry weight is similar to that presented by Romanoff (1949) (see also Romanoff, 1930; and Romanoff & Romanoff, 1933). The increase in wet weight during the early stages of incubation is due to the absorption of fluid from the white, i.e. to the formation of sub-blastodermic fluid as described by New (1956). The subsequent decrease in both wet and dry weight is arrested at about 14 days and these values are relatively constant between 14 and 18 days of incubation.

The protein nitrogen of yolk decreases to a minimum (about 65 per cent. of the original amount) after 14 days' incubation and subsequently rises almost to the initial value at 16–18 days (Text-fig. 1). Thereafter the level drops to about

40 per cent. in the hatched chick (i.e. after 21 days' incubation). The peak in protein content of the yolk is even more pronounced when eggs from any one bird are considered. A drop in the protein phosphorus content of the yolk is not

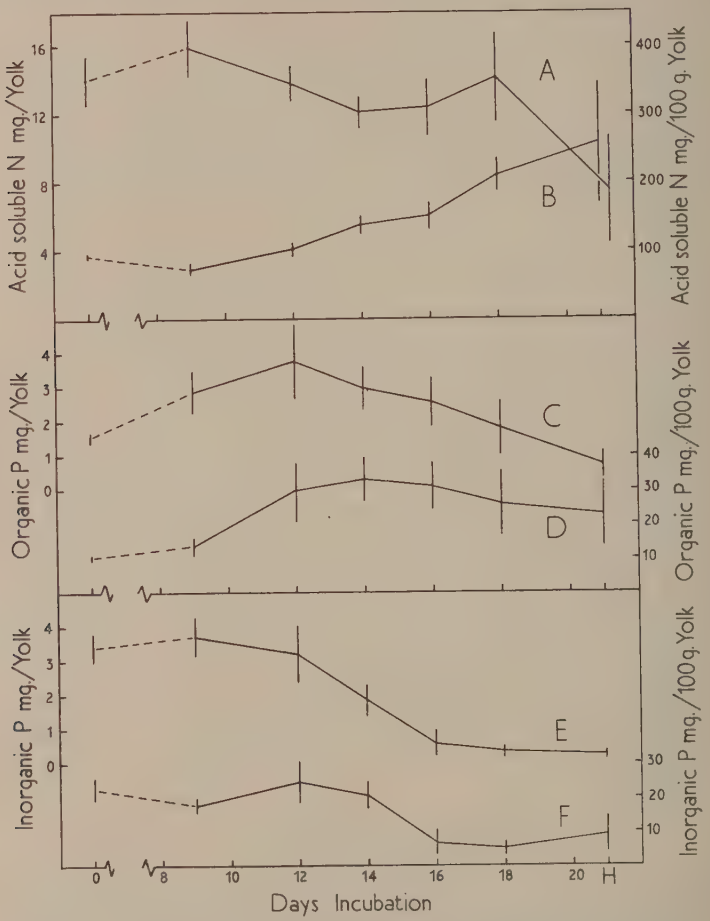


TEXT-FIG. 1. Changes in composition of yolk during chick embryogenesis. A, wet weight; B, dry weight; C, protein nitrogen; D, protein phosphorus. Results, expressed as amount per yolk, are for eggs from a group of eight hens. The vertical lines determine the limits of the standard deviation.

apparent until after 9 days' incubation and then more than half disappears by 14 days. The phosphoprotein decreases exponentially at a rate of about 20 per cent. per day until very little remains at hatching.

In addition to the above observations, estimates of both the concentration and absolute amount of acid-soluble nitrogen and phosphorus have been made (see

Text-fig. 2). The concentration of acid-soluble nitrogen progressively increases after 12 days' incubation. (Flickinger, 1957, and Williams *et al.*, 1954, have observed earlier increases in the concentration of free amino-acids.) However,



TEXT-FIG. 2. Changes in the acid-soluble components of yolk during chick embryogenesis. Acid-soluble nitrogen: amount per yolk, A; concentration, B. Acid-soluble organic phosphorus: amount per yolk, C; concentration, D. Inorganic phosphorus: amount per yolk, E; concentration, F. Results are for eggs from a group of eight hens. The vertical lines determine the limits of the standard deviation.

the amount of acid-soluble nitrogen per yolk remains fairly constant until near the end of incubation. Acid-soluble organic phosphorus increases in amount and concentration to maximum values in the 12- to 14-day period. Subsequently it

decreases, especially in amount per yolk. In contrast, inorganic phosphorus varies little until about 14 days, when it drops to a low level.

DISCUSSION

Yolk protein phosphorus decreases rapidly during the latter half of incubation. The results obtained by Holoubek & Brada (1956) for Wyandotte eggs follow a similar pattern, though the decrease they observed is less dramatic. When the major part of the phosphoprotein is disappearing (about 10–14 days) the amount of acid-soluble organic phosphorus of the yolk is greater than at other times, whereas the inorganic phosphorus remains at the original level. This suggests that the phosphoprotein may be broken down to phosphopeptides and phosphoserine before the liberation of inorganic phosphate. The reverse appears to be true of amphibian eggs in which a phosphoprotein phosphatase is present (Harris, 1950; Flickinger, 1956; Nass, 1956). Foote & Kind (1953) could not detect phosphoprotein phosphatase in the yolk of incubated hen eggs though it was found in the yolk-sac tissue.

After 15 days' incubation the acid-soluble organic phosphorus decreases, and inorganic phosphorus drops to a low value. It is during these last few days of incubation that the greater part of yolk phospholipid is assimilated and the rate of assimilation increases as incubation proceeds (Tsuji, Brin, & Williams, 1955; Holoubek & Brada, 1956; McIndoe, unpublished).

It is assumed, or suggested, in the general literature that some egg white passes into the yolk sac during embryogenesis. For example, Needham (1931) writes: 'it is known that the contents of the albumen sac tend to be included in the yolk at the end of development, about the time of the opening of the sero-amniotic duct' (see also Needham, 1954), and Hamilton (1952), referring to the experiments of Hanan (1927) with dyed egg white, says 'this method also demonstrates that albumen may pass into the yolk sac through the yolk sac umbilicus'. There is little evidence to support these statements in the original literature. Schenck (1932) suggested that a considerable amount of albumen was assimilated via the yolk towards the end of incubation, but his data are very limited and apparently derived from eggs of different hens.

In the present work the marked increase in the protein content of the yolk, which begins prior to the 16th day, supports the above contention, and the data suggest that about 30 per cent. of the total egg-white protein enters the yolk sac. It is possible that this assimilation begins before the 14th day. It may be noted that the increased level of protein in the yolk coincides with the temporary arrest in the decreasing wet and dry weight. It is possible that the limited capacity of the amnion to take up albumen, the pressure exerted by the growing embryo, and the slight negative pressure which probably exists in the yolk sac, all operate to force some egg white into the latter.

If eggs from birds taken at random had been examined, a less well-defined

pattern than that observed here would probably have emerged, and this may be the explanation of the results of Rupe & Farmer (1955), who examined the amounts of individual amino-acids in yolk up to 400 hours' incubation and who did not demonstrate an initial fall followed by a rise. Instead, a general decrease towards the end of the incubation period was observed, but it is significant that the amino-acids relatively more abundant in egg white proteins (i.e. methionine, phenylalanine, glutamic acid, and tryptophan) showed the least decrease after 400 hours' incubation.

SUMMARY

1. Changes in the wet and dry weight, protein nitrogen, and protein phosphorus content of yolk have been examined during chick embryogenesis. Observations on the acid-soluble nitrogen and phosphorus have also been made.

2. Protein nitrogen decreases to a minimum at about 14 days' incubation and rises again almost to the value for unincubated eggs at 16–18 days; subsequently it falls to half the original value at hatching. It is suggested that approximately 30 per cent. of egg-white protein is assimilated via the yolk sac during the last week of incubation.

3. After 9 days protein phosphorus decreases exponentially until very little remains at hatching.

4. During the period when the greater part of the phosphoprotein is being degraded (10–14 days) the amount and concentration of acid-soluble organic phosphorus in the yolk is high.

5. The concentration of acid-soluble nitrogen increases progressively after 12 days.

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The Distribution of Sulphur in the Differentiating Visceral Cartilage of *Xenopus*

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WITH ONE PLATE

INTRODUCTION

DURING embryonic development the differentiation of different tissues depends largely on the synthesis of specific substances characteristic of each tissue. From this viewpoint it is of interest to study the uptake of sulphur by the early embryo, especially since the incorporation and retention of this isotope in sulpho-mucopolysaccharides has now been well established by various authors working on fully differentiated tissues (see review by Dziewiatkowski, 1958).

So far some work has been done on the distribution of radiosulphate in early embryos (Amprino, 1955 *a*, *b*; Friberg & Ringertz, 1956; Johnston & Comar, 1957), but for amphibians in particular no information is yet available. The present paper deals with the incorporation of radiosulphate in various embryonic tissues of *Xenopus*, in particular in the visceral cartilage of ectomesodermal (neural crest) origin.

MATERIAL AND METHODS

Embryos of *X. laevis* in stages 29–47 (Nieuwkoop & Faber, 1956) were used. Carrier-free sulphate-S^{35} (specific activity about 6 c./mg. S) was added to full-strength Holtfreter's solution at 5 μc./c.c. The medium also contained 0·05 per cent. sulphadiazine (May & Baker). All cultures were maintained at room temperature.

Two types of experiments were performed:

(1) Anterior halves of *Xenopus* embryos in various stages of development, transected behind the gill-bud, were transferred into the radioactive medium for 4 or 6 hours. The epidermis was still unhealed at the end of culture.

(2) Small fragments consisting of the three germ-layers, removed from the

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gill-bud region of embryos in stage 29/30, were cultured as explants. The explants were cultured in radioactive medium immediately after excision or after 2, 4, or 6 days' explantation in non-radioactive medium. As the explants were completely covered by epithelial tissues (ecto- or endo-dermal) some time after explantation, they were split open just before being cultured in order to facilitate penetration by the tracer molecule. The explants had healed when removed from the radioactive medium, generally after 3 hours.

Fixation was carried out immediately after labelling or after a further culture for 2 or 4 days in non-radioactive medium. After thorough rinsing the specimens were fixed in Bouin, sectioned at 5 μ, and bleached when necessary with lithium carbonate. Autoradiographs were prepared with Kodak AR. 10 film. After exposing for 30 or 45 days and processing, the preparations were stained with methyl green-pyronin or toluidine blue. The latter stained either ortho- or metachromatically. The strength of labelling was assessed by subjective estimation, the range of autoradiographic densities being ample enough to justify this procedure. Under the resolution conditions of the preparations it is impracticable to distinguish between labelling of the peripheral and of the more central cytoplasm, so that 'labelled cytoplasm' may refer to one or both.

RESULTS

Observations on the whole head

Conspicuous differences exist in the uptake of sulphate by the various tissues in different stages of development. The main points will be given in the order of developmental stages.

Stage 29/30. Considerable uptake was observed at this stage. Although the distribution of tracer is more uniform than in later stages, mesodermal derivatives (undifferentiated mesenchyme of ecto- and endo-mesodermal origin, and heart) show an uptake a little higher than other tissues. As to the ectodermal derivatives, the white matter of brain and the ear showed fairly high uptake.

Stage 40. At this stage the various ectomesodermal elements later to give rise to visceral cartilage were distinguishable as mesenchymal condensations (precartilage). These future cartilage cells picked up particularly large amounts of sulphate, which was found in the cytoplasm and mainly in the intercellular space, while its presence in nuclei was doubtful. No metachromatic substance was detected in the intercellular space. The uptake in the other tissues was as mentioned for the earlier stage.

Some heads labelled at stage 40, and fixed after 2 or 4 days' culture in non-radioactive medium, were morphologically very much distorted and their development was delayed as compared with that of intact embryos, but the cells were still quite healthy. By the time of fixation the cartilage matrix was prominent. After 2 days, in spite of a general decrease of radioactivity in these heads, the cartilaginous tissue retained as much (or even more) tracer as the heads

fixed immediately after labelling. In this tissue the tracer was in the cytoplasm and the matrix. Especially in the matrix, which showed faint metachromasia, the amount of tracer was higher relative to that in the cytoplasm than in precartilages fixed immediately after labelling. This migration of tracer was still more pronounced after 4 days of culture.

Stages 45–46. The uptake was very high in the visceral cartilages, which were in an early stage of differentiation and with metachromatic intercellular matrix. The tracer was in both cytoplasm and matrix, whilst none or little was in the nucleus and nuclear membrane (Plate, fig. A). The labelling of matrix relative to that of cytoplasm increased from the 4th to the 6th hour of stay in tracer. The matrix was still too thin for internal differences in tracer content to be resolved by the autoradiographs. The future cartilage of the auditory capsule and the prechordalia were still at the stage of condensed precartilage and their uptake was less than that in visceral cartilages, except for a restricted area of the auditory capsule, assumed to be the centre of chondrification, which showed a high uptake.

No difference from the earlier stages was generally detected in tissues other than cartilage. One exception was the pharyngeal mucosa, which showed a fairly high uptake and metachromasia. Metachromatic substance appearing in the intercellular space of mesenchyme was accompanied by a little uptake.

Stage 47. In this stage much cartilaginous ground substance was deposited in the visceral skeleton, and the uptake by this tissue is very conspicuous (Plate, fig. B). The distribution of tracer was very characteristic in that it was especially abundant in the matrix newly deposited at the periphery of the chondrocytes. Other parts of the matrix and cytoplasm incorporated much less sulphur and none was detectable in the nucleus (Plate, fig. C). The amount of sulphur in the matrix, relative to that in the cytoplasm, was greater after 6 than after 4 hours of labelling.

Observations on explants

The differentiation of the explants was quite similar to what it would have been *in vivo* for the explanted tissues, i.e. they developed pharyngeal endoderm, cartilage, mesenchyme, muscle-cells, and epidermis. As the development was very much delayed in the explants, cartilaginous tissue in its early stage of differentiation did not appear before the 6th day of culture. When the labelling took place during this late stage of differentiation, uptake of tracer was very much higher in this tissue than in others and it occurred mainly in the matrix and cytoplasm. When labelling took place during the earlier stages of differentiation, the distribution of sulphate was rather uniform, though perhaps the amount in the undifferentiated mesodermal elements was a little greater than elsewhere.

Two explants labelled during the 4th day of explantation were fixed after a further 2 days' culture in non-radioactive medium. The uptake in newly differentiated cartilages was higher than in the undifferentiated mesodermal cells in the

explants fixed immediately after labelling, whilst other tissues had lost the tracer, thus increasing the contrast in tracer content between chondral and non-chondral elements. The intercellular matrix, which had not yet appeared at the time of culture in tracer, was well labelled.

DISCUSSION

So far the distribution of radiosulphate in early embryos has been recorded for the chick (Amprino, 1955 *a*, *b*; Johnston & Comar, 1957) and the rat (Friberg & Ringertz, 1956). Particularly high uptake by cartilaginous tissue has usually been demonstrated. The present results extend these observations to the developing amphibian embryo. In the explanted tissue fragments, as well as *in toto*, a higher uptake in mesodermal (ecto- and endo-mesodermal) mesenchyme is already recognizable in the undifferentiated stage, to become very conspicuous in the differentiating cartilages.

In early stages, when no matrix has yet been deposited in the intercellular space, the tracer is mainly in the cytoplasm of precartilage cells. At the onset of matrix deposition the intercellular space shows metachromasia and both cytoplasm and matrix become strongly labelled (see Plate, fig. A). In the fully differentiated cartilages the tracer is found mainly in the matrix and especially in the immediate periphery of the chondrocytes (see Plate, figs. B, C). Our observations on the shift of relative concentrations of tracer from the chondrocytes to the matrix in (*a*) the cartilage of stage 40, kept from 2 to 4 days in normal medium after labelling, (*b*) the cartilage of stages 45–46 and 47, from the 4th to the 6th hour of labelling, and (*c*) the explants cultured for 2 days after labelling, all indicate that sulphate is first stored in the chondrocytes before being released in the matrix. Other authors have reached similar conclusions on fully differentiated cartilage (Pelc & Glücksmann, 1955; Fell, Mellanby, & Pelc, 1956; Mancini, Núñez & Lustig, 1956; Johnston & Comar, 1957). Nearly the same observations were also obtained in the cartilage of vertebrae and extremities (both of endomesodermal origin) of chick and mouse embryos differentiating in organ culture (Okada, 1959, and unpublished data). It can then be concluded that the differential localization of sulphur in the different stages of cartilage histogenesis is general to both the ecto- and endo-mesodermal cartilages of the vertebrate embryo.

The intercellular accumulation of sulphur revealed by the autoradiographs shows parallelism with the appearance of metachromasia in these sites. According to the evidence presented by other authors (Dziewiatkowski, 1951; Boström, 1952; Boström & Månsson, 1952), it is valid to consider that the intercellular deposition of sulphur is in the form of chondroitin-sulphate. On the other hand, the precise chemical form in which the sulphate is found in the cytoplasm of precartilaginous cells is not yet known. It was suggested, however, that in precartilaginous cells of the chick embryo the future mucopolysaccharide component of the intercellular matrix is already demonstrable as PAS-positive

granules inside the cells (Moscona & Moscona, 1952). At any rate, it is now quite certain that these future cartilaginous cells establish their specificity in sulphate metabolism prior to their visible differentiation (present observations; and for the chick embryo those of Amprino, 1955*b*; Johnston & Comar, 1957; and Okada, 1959).

The question necessarily arises whether the specificity revealed by sulphate metabolism foreruns the so-called 'determination' of the future cells of visceral cartilage. It is known that the determination of visceral cartilage cells does not occur in the neural crest of the neurula, and that the cartilage differentiation of this material can be realized only under the inductive influence from endoderm and sometimes from notochord (Newth, 1954; Wilde, 1955; E. W. Okada & Ichikawa, 1956). But explantation experiments on *Rana japonica* indicate that the ecto- and endo-mesodermal layers of the gill region taken from tail-bud stages (approx. stage 29/30 in *Xenopus*) can differentiate into cartilage in the absence of both endoderm and notochord (E. W. Okada & Ichikawa, unpublished). In contrast, according to our results the precartilaginous cells first indicate their specificity by stage 40. Comparing the conclusions from these two different experimental approaches it is clear that the 'determination' detected by purely embryological tests precedes the establishment of metabolic specificity in these cells, but the latter foreruns the visible differentiation of chondroblasts.

SUMMARY

1. The incorporation of sulphate into tissues of *Xenopus* embryos in various developmental stages was studied by means of autoradiography.

2. Particularly high uptake was demonstrated in the precartilages as well as in the differentiated cartilages.

3. In the earlier stages, when no intercellular matrix has yet been deposited, the tracer is mainly in the cytoplasm of precartilaginous cells, while it is mainly in the matrix and especially in the immediate periphery of the chondrocytes of the differentiated cartilaginous tissue.

4. The movement of tracer stored in the cytoplasm to the intercellular matrix accompanies the histogenesis of cartilage.

ACKNOWLEDGEMENT

We are indebted to Professor C. H. Waddington for his interest. One of us (T. S. O.) held a Macaulay Fellowship from the University of Edinburgh, which made his work in the United Kingdom possible.

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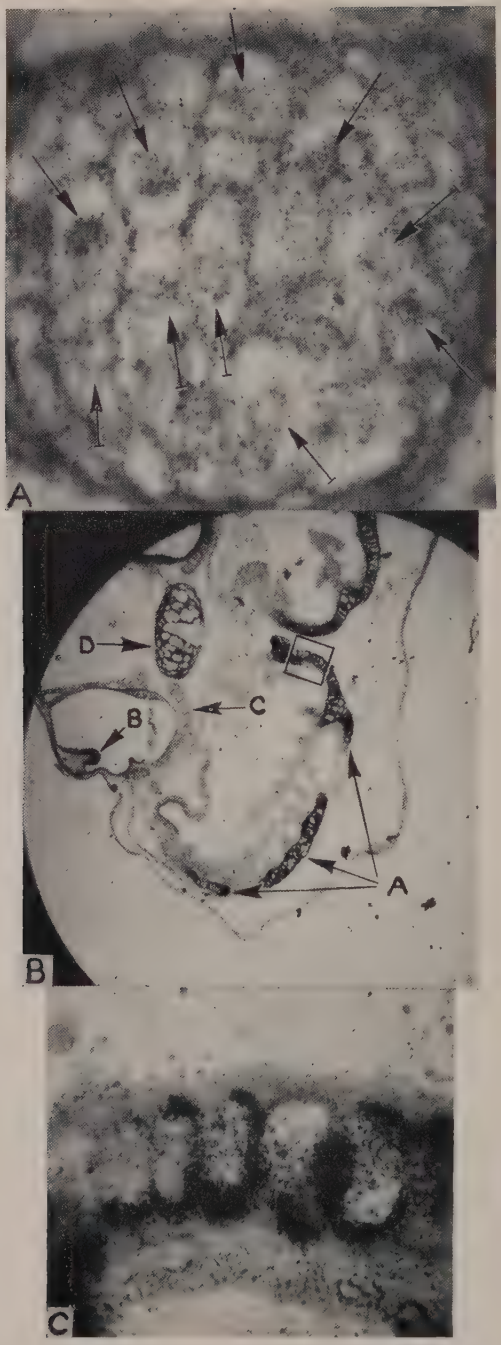
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EXPLANATION OF PLATE

Fig. A. Basibranchial cartilage of a stages 45–46 embryo. The strength of autoradiographs is about equal in cytoplasm and cartilage matrix. Cell-bodies (retracted from matrix) indicated by arrows. Five arrows with heel indicate cells in which the autoradiograph is seen to originate in the cytoplasm and not in the nucleus. ×800.

Fig. B. Stage 47 embryo. Strong autoradiographs on visceral (branchial) cartilages (A) and presumed centre of chondrification in the auditory capsule (B). Weaker autoradiographs on cartilage of the auditory capsule (C) and chorda (D); in the latter the photographic contrast is due to stain and not to an autoradiograph. Inset magnified in fig. C. ×60.

Fig. C. Inset from fig. B. magnified. Intense autoradiographs on parts of the cartilage matrix in the immediate vicinity of the chondrocytes, and no autoradiographs on the more distant matrix. Much weaker autoradiographs on the chondrocytes. ×900.

(Manuscript received 7 : vii : 59)

Comparative Investigations of the Action of Two Nitrogen Mustard Derivatives on the Early Stages of Development of Chick Embryos

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WITH TWO PLATES

CYTOTOXIC compounds act by combining with the biochemical constituents of cells. Because of the complexity of living matter, the cytotoxic activity is highly complicated in nature and is therefore far from being thoroughly understood. In order to analyse the cytotoxicity of any chemical compound, many biological variables concerned in determining the mode of action of the compound and its selectivity for any particular range of cells have to be taken into account (Danielli, 1952, 1954).

Cells of the early embryonic stages are a suitable material for cytotoxic investigations. Although not completely differentiated, they soon arrange themselves into a few embryonic tissues originating directly from the three fundamental germ layers. These tissues consist of cells which may be regarded as the precursors of all the cells of the adult organ. It is interesting to inquire whether they show in these early stages a specific selectivity to cytotoxic compounds which is similar to the selectivity of tumour cells, and which may later be derived indirectly from different germ layers.

One of many biological variables which should be taken into account in cytotoxic investigations is the presence or absence of particular enzymes causing the cells to be more sensitive or more resistant to the cytotoxic drugs concerned. So far, very little information is available on the enzymatic constitution of the germ layers (Steinbach & Moog, 1955). The experiments reported here aimed at comparing the cytotoxic activity of two related derivatives of nitrogen mustard, one of which is an acetyl derivative of the other. Their formulas are as follows:

$H_2N\text{-}C_6H_4\text{-}N(CH_2\text{-}CH_2\text{-}Cl)_2$	$H_3C\text{-}CO\text{-}NH\text{-}C_6H4\text{-}N(CH_2\text{-}CH_2\text{-}Cl)_2$
N-(*p*-amino-phenyl)-2,2′-dichloro-diethylamine ('parent compound')	N-(*p*-acetyl-amino-phenyl)-2,2′-dichloro-diethylamine ('acetyl derivative')

It has been shown that the acetyl derivative is relatively less toxic for rats, and

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that it is readily decomposed into its parent compound in tissues where hydrolytic peptidase is available (Danielli, 1954). This takes place particularly in cells of the Walker rat carcinoma, and the acetylation allows a much greater degree of selectivity in the cancerostatic properties. It seemed interesting to find out whether similar decomposition takes place in embryonic tissues and, if so, where and to what extent.

MATERIAL AND METHODS

Chick embryos at the definitive-streak stage (stage 4 according to Hamburger & Hamilton, 1951) explanted from eggs and cultured *in vitro* (New, 1955) were used. The chemical compounds were both obtained from Boots Drugs Co. Ltd., and were applied in a solution of liquid egg albumen containing 10 per cent. of sterile saline (0·9 per cent.). The concentrations of the two compounds are shown in the following table.

TABLE 1

		Number of embryos treated	
No.	*Concentration*	*Parent compound*	*Acetyl derivative*
1	4×10^{-6}	6	5
2	8×10^{-6}	5	4
3	$1{\cdot}6\times10^{-5}$	6	6
4	$3{\cdot}2\times10^{-5}$	6	7
5	$6{\cdot}4\times10^{-5}$	27	21
6	10^{-4}	23	26
7	$1{\cdot}28\times10^{-4}$	8	6
8	$1{\cdot}5\times10^{-4}$	32	35
9	2×10^{-4}	10	16
10	5×10^{-4}	8	12
11	10^{-3}	4	4

In all the experiments the solutions were administered in amounts of 0·5 c.c. put round the plastic ring used. In the control experiments liquid egg albumen containing 10 per cent. of physiological saline was used. Reincubation of both control and experimental embryos lasted about 24 hours, until the control embryos had developed 15–19 somites, i.e. to stages 12–13 according to Hamburger & Hamilton (Plate 1, fig. 1).

Carnoy's liquid was used as a fixative. For macroscopical examination fixed embryos were stained with Carmalum or Mayer's haematoxylin and prepared as whole mounts. For histological and cytological purposes embedding was performed by standard methods. Transverse or longitudinal sections 6 μ thick were stained with methyl green-pyronine or in Feulgen reagent.

RESULTS

At concentrations lower than $6{\cdot}4\times10^{-5}$ (nos. 1–4 in Table 1) neither the parent compound nor the acetyl derivative affected the external appearance or

the microscopical structure of the treated embryos. Higher concentrations (nos. 5–9) of either compound proved to be cytotoxically effective. There was no significant difference between the compounds in degree of cytotoxicity. At the last two concentrations (nos. 10 and 11) both compounds appeared to be highly toxic, causing detachment, shrinkage, and necrosis of the blastoderms.

Macroscopical observations

After the lowest effective doses (nos. 5 and 6) the experimental embryos were found to be at stages 10 or 11, whereas the controls were at stages 12 or 13. Retardation of development became more evident as the concentration increased, being greater in some regions of the embryo than in others. The most severely affected organs appeared to be the paraxial mesoderm, the somites, and the neural tube.

Macroscopical observations indicated that the axial mesodermal structures were the most sensitive parts of the embryo. When solution no. 6 was used, both the parent compound and the acetyl derivative produced embryos with severely affected somites. A mild degree of change was characterized by complete emptiness of the somites, due to the absence of the somite core, and a considerable widening of the myocoele, which made the somites appear to consist of ring-shaped accumulations of mesodermal tissue. Along with this abnormality there was observed a marked shortening of the longitudinal axis of the body as compared to the control embryos. This was due more to the changes in the somite region than to those in the head part (Plate 1, figs. 2, 3).

Further changes, observed after treatment with concentrations nos. 7 and 8 of both compounds, were severe disturbances in the shape and arrangement of the somites, which were much fewer in number and far less dense in structure than those in the control embryos (Plate 1, figs. 4, 5). After higher concentrations, and especially after concentration no. 9, there were found embryos with almost completely disintegrated somites, or with only a few vestigial groups of mesodermal cells in the somite region bearing little resemblance to somites in their arrangement (Plate 1, fig. 6).

In all effective concentrations both compounds frequently caused the paraxial mesoderm to move away from the axial line in the region of the sinus rhomboidalis caudal to the somite region, leaving free areas on both sides. In some embryos there were characteristic symmetrical patterns of defective structure in the paraxial mesoderm (Plate 1, figs. 6, 7, 8, 9).

The neural tube was affected by both substances in the same manner. After concentrations nos. 7, 8, and 9 the brain roof usually remained unclosed (asyntaxia dorsalis), while the rest of the neural tube was unclosed in some parts or, in extreme cases, throughout its entire length (Plate 1, figs. 7, 9).

There were some differences in appearance between heads of embryos treated with the parent compound and those treated with the acetyl derivative. After doses nos. 5 and 6 of the parent compound the heads, although nearly normally

developed, appeared to be much narrower than those of embryos treated with the corresponding solutions of the acetyl derivative, which did not differ very much from the heads of control embryos. Although deprived of some somites and with partially unclosed neural tubes, the heads of these embryos treated with the acetyl derivative appeared almost normal and had mesenchyme cavities filled with the normal quantity of head mesenchyme (Plate 1, figs. 4, 7).

Microscopical observations

After treatment with both compounds, in cytotoxically effective concentrations chosen from the range used in preliminary experiments, the neural tube (i.e. brain and spinal cord), the somites, and, to some extent, the mesenchyme showed pronounced changes in their cytological structure.

The neural tube, after treatment with both compounds in the effective concentrations, showed degenerating cells of various types depending on the concentration used. In embryos treated with concentrations nos. 5–7, both compounds caused an increase in the number of neural tube cells containing nuclei 2–3 times larger than normal, with similarly enlarged nucleoli (Plate 1, figs. 10, 11). Nuclear enlargement, whether slight or very pronounced, appeared to be the first sign of the cytotoxic activity of these compounds. The enlarged nuclei showed poor stainability with methyl green, as can be seen in Plate 1, fig. 11, in which the enlarged nuclei appear to be almost colourless. Experiments using the Feulgen reaction proved, however, that the DNA content of these nuclei did not differ from that of control cells, except that it was more dilute due to the nuclear enlargement.

In embryos treated with the more concentrated solutions (nos. 8 and 9) there were, besides the changes in the size of the cell components, many neural tube cells showing necrotic changes such as karyorrhexis, pycnosis, and nuclear disintegration. Nevertheless, in the immediate neighbourhood of the degenerating cells there were found cells in different phases of cell-division. In the cytoplasm of the degenerating cells there were often found large and fine pyronine-positive granules stained like the nucleoli (Plate 2, figs. 12, 13).

Simultaneously with the changes on the cellular level the neural tube tissue showed increased retardation of its organogenetic development, particularly as regards its closure, as the degenerative changes in the cells became more pronounced.

Microscopical investigations of the somite region in embryos treated with both compounds showed that, in general, these structures are extremely sensitive to increases in the concentrations of the cytotoxic agents. Comparing figs. 14, 15, 16, and 17 of Plate 2 (demonstrating the histological structure of corresponding somites in longitudinal section) one can see the associated stages of degeneration in these important embryonic organs following treatment with different concentrations of the compounds. The more severely affected somites fail to show any differentiation into sclerotome, dermatome, and myotome. In such cases they

represent merely unorganized cell aggregations showing almost no metamerism and are sometimes fused into irregular lumps of mesodermal tissue.

In the experimental embryos examined the somite cells showed changes similar to those in the neural tube cells, although they did not undergo the extreme degenerative processes observed commonly in the latter. The enlargement of the somite cells and of their internal constituents was usually associated with a more or less marked disintegrating effect in their organogenetic appearance.

Other mesodermal structures (heart and extraembryonic mesoderm) did not show any other cytological changes than those described in the somites. Heart anlage, along with the general retardation of development, was usually also retarded. The blood islands showed a normal structure and contained similar numbers of dividing cells to those found in the control embryos.

The head mesenchyme also underwent degenerative changes, which were more noticeable after treatment with the parent compound than after the acetyl derivative. The differences are shown in Plate 2, figs. 18, 19, 20. After treatment with the parent compound the head mesenchyme tissue became less dense and its cells appeared slightly larger, with enlarged nuclei and nucleoli. Their cytoplasm was often vacuolized and their external shape was in most cases spherical, without the usual amoeboid character. These morphological changes suggest loss of the ability of the cells to form pseudopodia and, therefore, defective motility (Plate 2, figs. 21, 22). In extreme cases, after administration of the parent substance in the highest effective concentration (no. 9), the head mesenchyme was almost completely absent and the head mesenchyme cavities were shrunken.

Treatment with the acetyl derivative in similar concentrations did not have any distinct influence on the number or cytological appearance of the head mesenchyme cells. Even after treatment with the highest concentrations they remained similar to the control embryos.

The endodermal cells of the fore-gut and archenteron roof appeared not to be easily influenced by either compound. A comparison of the severely affected neural tube cells with the endodermal cells of the same embryo demonstrated the greater resistance of the latter.

Similarly, the notochord cells were more resistant to the cytotoxic activity of both compounds than were the neural tube and mesodermal cells.

DISCUSSION

According to data given by Danielli (1954, 1959) the LD_{50} (rats) of the parent compound is 6–8 mg. per kg., whereas that of the acetyl derivative is 48–50 mg. per kg. This means that in rats the former compound is roughly 7 times more toxic than the latter.

In the experiments reported here it was found, however, that there was no difference between the two compounds in the effective and toxic doses. This fact seems to suggest that the cytotoxic mechanism of these compounds in embryonic tissues is in some way different from that in the adult rat. If it is true that the acetyl derivative acts cytotoxically after being enzymatically activated, due to hydrolysis by some kind of peptidase, and that it does not act as a whole molecule, it can be assumed that in chick embryos it is decomposed in all the cells except those of the head mesenchyme.

In all the affected tissues degenerative changes were observed. After the lower concentrations the affected cells underwent either slight or severe cytoplasmic, nuclear, and nucleolar enlargement, sometimes reaching as much as 3 times their normal size. At higher doses they showed, in addition, karyorrhexis, pycnosis, and nuclear fragmentation. Similar degenerative effects have been recorded after nitrogen mustard in chicks (Karnofsky, 1950), after triethanomelamine in amphibian embryos (Waddington, 1958) and in chick and mouse embryos (Jurand, 1958, 1959), and after many different antimetabolites and antagonists (Waddington, Feldman, & Perry, 1955; Schultz, 1959).

Changes in somite differentiation have been observed in chick embryos after treatment with amino-acid analogues (Rothfels, 1954; Herrmann, Königsberg-Rothfels, & Curry, 1955) after purine antagonists (Waddington, Feldman, & Perry, 1955), and triethanomelamine (Jurand, 1958, 1959). There is a suggestion that all these changes, induced in such different ways, are due to some not fully understood disturbances in protein synthesis which give rise to the abnormal appearance and the loss of differentiation capacity of the affected tissue.

After treatment with either of these compounds cells at various mitotic stages are always found in the immediate neighbourhood of cells in the process of degeneration. This suggests that neither the parent compound nor its acetyl derivative can be regarded as having antimitotic properties.

The difference between the marked sensitivity of the neural tube, mesodermal structures, and head mesenchyme cells on the one hand, and the resistance of the entoderm and notochord on the other, is striking. There is no clear explanation of this difference. It is known that nitrogen mustard and its derivatives, as well as other related compounds, sometimes have an effect in the case of neoplastic diseases of blood-forming organs that are of mesodermal origin; but in the embryos neural tissue appears to be as sensitive as the axial mesoderm, and there is no evidence for any strong effect on the blood-islands.

The only difference found between the two compounds in the experiments reported here was that the parent compound affects the head mesenchyme cells whereas the acetyl derivative appears to have less effect on this tissue. A possible explanation is that it is due to the low content of hydrolytic protease in the head mesenchyme cells, but, so far, no proof of this has been obtained by cytochemical or histochemical investigations.

SUMMARY

1. N-(*p*-amino-phenyl)-2,2′-dichloro-diethylamine and its acetyl derivative have the same degree of toxicity for early stages of chick embryo development.

2. In concentrations of $6{\cdot}4 \times 10^{-5}$ to 2×10^{-4} they cause degeneration of the neural tube and somite cells. The first sign of degeneration is the enlargement of the affected cells. More severe damage (karyorrhexis, pycnosis, and nuclear disintegration) leads to pronounced disturbances in the differentiation of the affected organs.

3. The parent substance causes degeneration of the head mesenchyme, whereas its acetyl derivative is much less effective in this tissue.

4. Both compounds have comparatively little effect on the endodermal structures, the notochord, the heart, or the lateral mesoderm.

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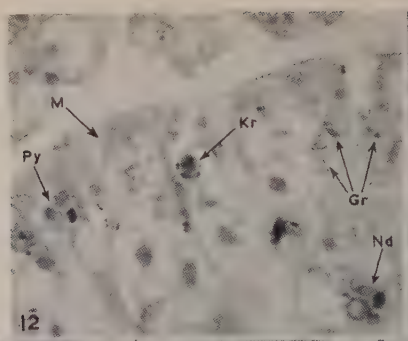
A. JURAND

Plate 1



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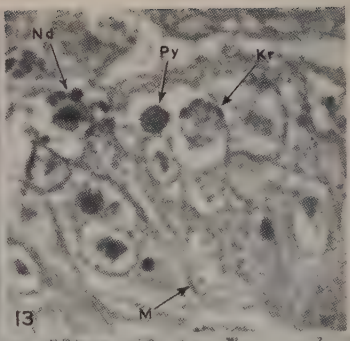

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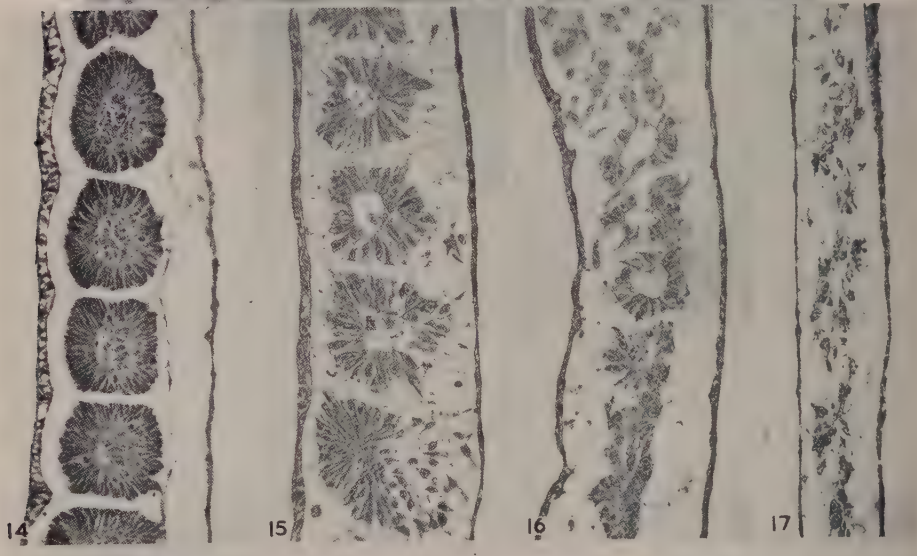

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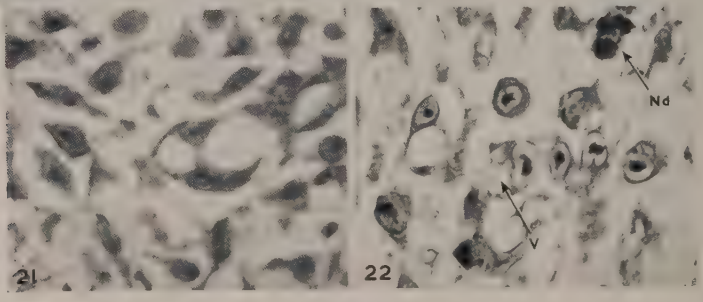

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A. JURAND

Plate 2

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EXPLANATION OF PLATES

PLATE 1

FIG. 1. Macroscopical view of a control embryo of 15-somite stages. ×20.

FIG. 2. An embryo treated with the parent compound (concentration $6{\cdot}4 \times 10^{-5}$). Note a marked shortening of the body-length. ×20.

FIG. 3. The somite region of the same embryo as Fig. 2. Note ring-shaped somites. ×50.

FIG. 4. An embryo treated with the acetyl derivative ($1{\cdot}28 \times 10^{-4}$). Note the decreased number and the less dense structure of somites. ×20.

FIG. 5. An embryo treated with the parent compound ($1{\cdot}28 \times 10^{-4}$). Note irregular arrangement of somites. ×20.

FIG. 6. An embryo treated with the acetyl derivative (2×10^{-4}). Note nearly complete lack of somites. ×20.

FIG. 7. An embryo treated with the parent compound ($6{\cdot}4 \times 10^{-5}$) showing comparatively normal development, but narrow head and unclosed neural tube in its caudal end. ×20.

FIG. 8. An embryo treated with acetyl derivative ($1{\cdot}5 \times 10^{-4}$). Note nearly complete lack of somites, destructive changes in the paraxial mesoderm, unclosed neural tube, but almost normally developed head. ×20.

FIG. 9. An embryo treated with the parent compound ($1{\cdot}5 \times 10^{-4}$). Note unclosed neural tube, vestigial somites, and the symmetrical pattern of the destructive changes in the caudal mesoderm. ×20.

FIG. 10. Neural tube cells of a control chick embryo at 15-somite stage. ×500.

FIG. 11. Neural tube cells of an embryo treated with the parent compound (10^{-4}). ×500.

PLATE 2

FIG. 12. Neural tube cells of an embryo treated with the parent compound ($1{\cdot}5 \times 10^{-4}$). *Py*, pycnosis; *Kr*, karyorrhexis; *Nd*, nuclear disintegration; *Gr*, pyronin positive granules in the cytoplasm; *M*, dividing cell. ×830.

FIG. 13. Neural tube cells of an embryo treated with the acetyl derivative ($1{\cdot}5 \times 10^{-4}$). *Py*, pycnosis; *Kr*, karyorrhexis; *Nd*, nuclear disintegration; *M*, dividing cell. ×830.

FIG. 14. Longitudinal section through somites of a control embryo. ×150.

FIG. 15. Longitudinal section through the somites of an embryo treated with the parent compound ($6{\cdot}4 \times 10^{-5}$). ×150.

FIG. 16. Longitudinal section through somites of an embryo treated with the acetyl derivative ($1{\cdot}28 \times 10^{-4}$). ×150.

FIG. 17. Longitudinal section through the somite region of an embryo treated with acetyl derivative (2×10^{-4}). Note the vestigial structure of the somites. ×150.

FIG. 18. Transversal section through the head of a control embryo. ×80.

FIG. 19. Transversal section through the head of an embryo treated with the parent compound ($1{\cdot}5 \times 10^{-4}$). Note the unclosed neural tube and loose mesenchyme tissue. ×80.

FIG. 20. Transverse section through the head of an embryo treated with the acetyl derivative ($1{\cdot}5 \times 10^{-4}$). Note nearly normal condition. ×80.

FIG. 21. Head mesenchyme cells of a control embryo. ×600.

FIG. 22. Head mesenchyme cells of an embryo treated with the parent compound. *V*, vacuolization of the cytoplasm; *Nd*, nuclear disintegration. Note the spherical shape of the cells. ×600.

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